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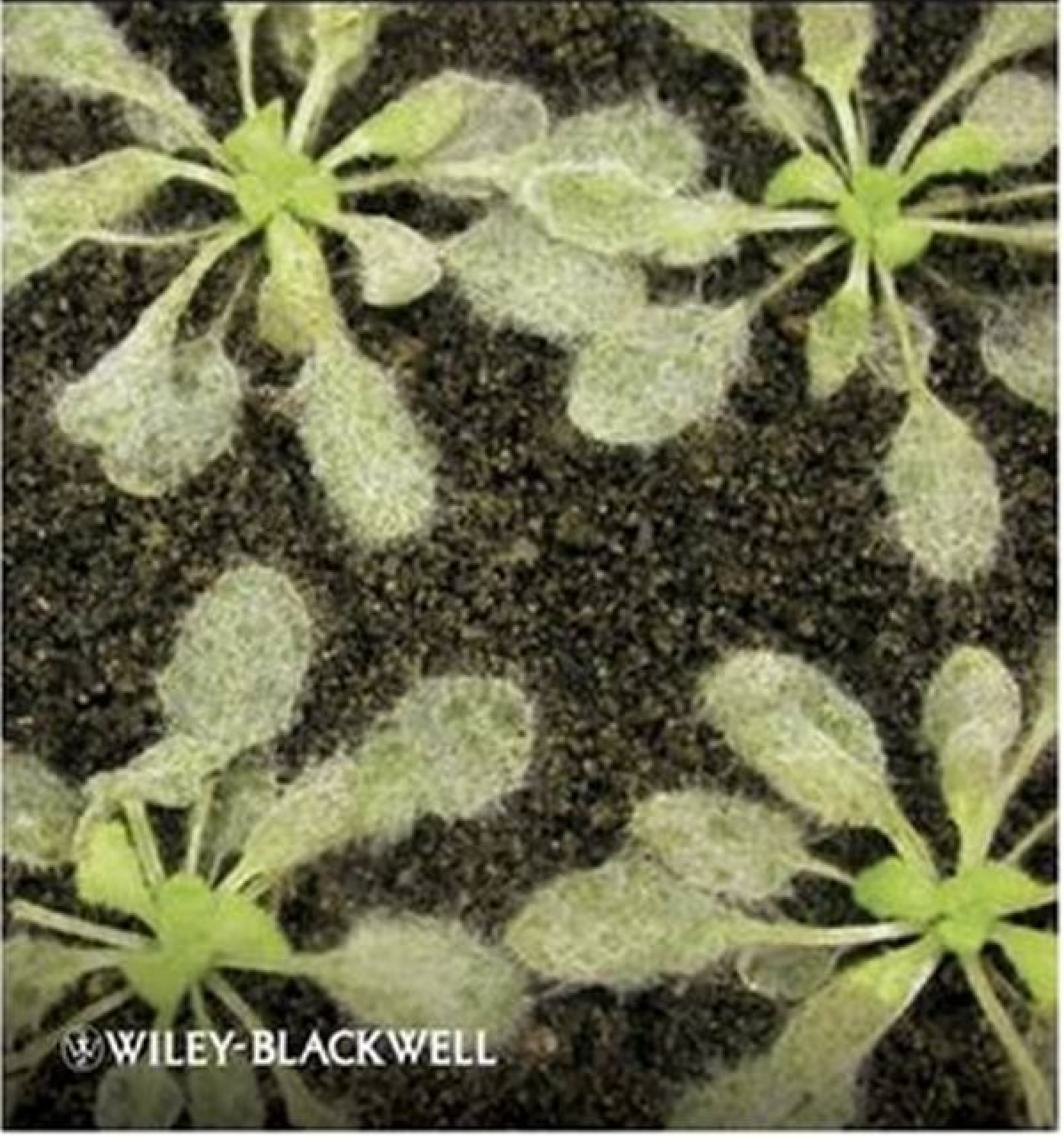
Molecular Aspects of Plant Disease Resistance

Edited by Jane Parker



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Molecular Aspects of Plant Disease Resistance

Edited by

Jane Parker

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PREFACE

Growing in their natural environments, the majority of plants remain healthy. There may be some flecking or leaf distortions, but most plants compete effectively and produce the next generation. This relative vigour is because plants have evolved an effective immune system that protects each of their cells from the majority of pathogens and pests. Only parasitic strains that have developed mechanisms to overcome multiple layers of plant innate immunity are able to invade and cause disease. Once disease takes hold it can be devastating, particularly in plant monocultures that have a narrow genetic base. The predicted increase in numbers of human beings having to survive on our planet, as areas of fertile soil to grow crops become more scarce and production costs soar, brings into sharp focus the need to understand better molecular processes of plant disease and plant disease resistance. The majority of the world's populations still battle against malnutrition, hostile environments and crop infestations. A heavy reliance on pesticides both in Western agricultural systems and in the developing world helps to maintain crop yields but is environmentally damaging and costly. The impetus has therefore never been stronger to comprehend better how pathogens evolve to infect particular plant species, how certain plant genotypes naturally defend themselves against pathogen infection and how plant disease resistance is executed at the level of individual cells, whole organisms and ecosystems.

In selecting themes for this *Annual Plant Reviews, Volume 34* I was like Richmal Crompton's young William Brown put in charge of Mr Moss's sweet shop – there were rich pickings! I have tried to capture some of the most dramatic advances made during the last 5–10 years in molecular aspects of plant–microbe interactions. For example, we now know that mechanisms determining plant disease susceptibility are inextricably connected to those governing plant defence. It is the fine balance of contrasting forces in the plant and invading pathogen that determines the outcome of these encounters. We can no longer view disease resistance signalling pathways in isolation without comprehending their relationship to other environmental stress response systems and phytohormone networks. The range of molecules produced by microbial pathogens, how they aid infection and are intercepted at different points by host cells is only now becoming properly appreciated as we obtain more genome sequence information on bacterial, fungal and oomycete pathogens and their plant hosts. Equally impressive is the diversity of plant secondary metabolites. There has been a renaissance in defining the structural range and activities of small molecules as key components of plant local and systemic response pathways. Also important is the spatial control of plant

resistance processes, now being revealed in intact cells and tissues using state-of-the-art microscopy and live cell imaging techniques. Knowledge of such fundamental processes underpins the development of new crop varieties created by modern molecular plant breeding and transgenic technologies.

There is a certain logic and progression to the chapters presented here. The first chapter is unusual in that it is a personal view of the changing landscape of molecular plant pathology over 40 years, presented by Emeritus Professor Michele Heath. It serves to highlight the momentum of research in this field and how ideas, paradigms and prejudices change over time. The next four chapters (2–5) deal with the important area of pathogen-derived molecules and effectors and how these interact with plant host cells to determine disease susceptibility or resistance. A recurring theme is the continual evolutionary battle between host and pathogen to gain control of defence activation thresholds. Chapter 6 describes emerging concepts in the most intimate of host–parasite interactions between plants and viruses. Particularly significant are gene-silencing systems that likely evolved as a barrier to foreign nucleic acid and are now known also to regulate many aspects of plant development. Chapter 7 covers the topic of spatial control of plant resistance mechanisms, emphasizing the dynamic nature of intracellular immune receptors and defence regulatory proteins in response to pathogens. This important dimension is extended in Chapters 8 and 9, which evaluate the roles of secondary metabolite pathways in plant–pathogen and plant–insect communication. The last two chapters (10 and 11) present our current understanding of local and systemic signalling processes and the importance of signal crosstalk in determining how plants respond to particular biotic stresses in the natural environment. A range of small molecules come to prominence, including lipid, phenolic and indole derivatives whose controlled mobilization is probably crucial for plant survival upon attack by pathogens and insects. Understanding the modes of action of such molecules, their systemic activities and the intricate chemical ecology of plant–pathogen–insect relationships has important implications for manipulating host survival and crop productivity in the field.

I thank all the authors for their excellent contributions and hope that this volume will be a valuable reference work for students and researchers in the area of molecular plant pathology.

Jane Parker

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Chapter 1

A PERSONAL PERSPECTIVE OF THE LAST 40 YEARS OF PLANT PATHOLOGY: EMERGING THEMES, PARADIGM SHIFTS AND FUTURE PROMISE

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Abstract: The last 40 years of experimental research have resulted in a remarkable increase in our understanding of plant disease resistance to microbial pathogens, with a recent surge of clarity primarily provided by the application of molecular genetics to pathogen interactions with *Arabidopsis thaliana*. Research foci have changed over time with the availability of new techniques and the ability to identify genes, proteins, signalling systems and defensive biochemicals involved in plant resistance. In hindsight, early concepts were generally simplistic. Although some have been supported by subsequent data, others, such as the basis for the gene-for-gene phenomenon, have changed dramatically and it is now clear that plant–microbe interactions are sophisticated and complex. Much is left to discover: the role of the hypersensitive response is still enigmatic, the interplay of recognition events and defensive factors that control host or non-host resistance is still not clear and the number of well-studied pathosystems is still few. The future promises more attention to the spatial organisation of disease resistance at the cellular level, and new insights into the evolution of disease resistance and pathogen pathogenicity. Particularly urgent is the need for unequivocal data to prove which plant genes and processes involved in disease resistance are primarily responsible for the restriction of pathogen growth. Disappointingly, our considerable progress in understanding plant–microbe interactions in the last 40 years has not translated into comparable progress in developing novel, widespread and effective methods of disease control in the field, and this remains a significant challenge for the future.

Keywords: disease resistance; gene-for-gene hypothesis; host resistance; hypersensitive response; non-host resistance; resistance genes

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1.1 Introduction

There is an often-quoted saying, of controversial origin and meaning, that states, 'May you live in interesting times'. Since entering the field of plant pathology as a graduate student in 1966, I believe that I have lived in very interesting times, both inside and out of academia. In 1966, the genetic code had only just been cracked and the field of 'physiological plant pathology' was in its infancy. In 1967, my PhD supervisor, Professor R.K.S. Wood, summarised virtually all that was known at the time about microbial infection and plant disease resistance in a 570-page book (Wood, 1967) – something that would be impossible to do today. When the book was being written, it was recognised that pathogens could produce disease-promoting substances, such as cell wall-degrading enzymes or toxins, that diseased plants had altered metabolism and that there was a variety of preformed structures and compounds that potentially could protect plants against pathogen attack. The more dynamic interactions between plant and pathogen were only just beginning to be appreciated with the discovery that plants actively produced low-molecular-weight antimicrobial compounds (phytoalexins) in response to infection (Cruickshank, 1963) and, subsequently, with the revelation that microorganisms might combat these compounds by producing phytoalexin-degrading enzymes (e.g. Higgins and Millar, 1970). Although there had been a number of earlier light microscopical investigations of diseased plants, in the 1960s, electron microscopy began to be applied to plant pathology, revealing new information almost daily about structural changes occurring at the plant–parasite interface, particularly with respect to fungal parasites (Bracker, 1967). It was an exciting time during the next two decades as more researchers became interested in the mechanisms of plant disease resistance, and models to explain host–parasite specificity were constructed and extensively debated as new biochemical, genetical and structural data were revealed (e.g. Hadwiger and Schwochau, 1969; Albersheim and Anderson-Prouty, 1975).

In hindsight, many of the early concepts of host–parasite specificity were simplistic, and there was a degree of arrogance in the assertions that some new technique or equipment would provide complete insight into how plant and pathogen were interacting. The progression of information and concepts since 1966 has been fascinating, and this review is a personal perspective of how concepts, paradigms and research focus have changed over the last 40 years in our search for an understanding of plant disease resistance. I have concentrated on three intertwined topics that, to me, best illustrate the often-tortuous route by which we have arrived at our current state of knowledge.

1.2 The hypersensitive response

The term 'hypersensitive' was first applied by Stakman (1915) to describe resistant cereals that responded to rust fungal infection with a rapid, but

limited, death of cells at the infection site. The subsequent realisation that an apparently similar rapid cell death was a common expression of plant disease resistance, both in resistant genotypes of host species and in non-host plants, led to its designation as the 'hypersensitive response' (HR). Whether the HR was a cause or consequence of disease resistance became a matter of hot debate (Heath, 1976). For biotrophic cellular pathogens or viruses, which require a living cell for growth, plant cell death at the infection site seemed a reasonable mechanism of disease resistance, but it was less obvious why the HR should be associated with resistance to non-biotrophic pathogens that were capable of growing in dead tissue. This conundrum seemed partially solved with the discovery that the HR appeared to be universally accompanied by antifungal phytoalexin accumulation at the infection site and that dead cells released molecules that elicited the synthesis of phytoalexins in their living neighbours (Hargreaves and Bailey, 1978). However, the picture became clouded again with the gradual appreciation that plants possess a multiplicity of inducible defensive factors in addition to phytoalexins, including other toxic molecules and a variety of antimicrobial proteins collectively known as pathogenesis-related (PR) proteins (van Loon and van Strien, 1999). The revelation that so many anti-microbial factors were associated with the HR and that these factors could be elicited by pathogen molecules in the absence of cell death, again raised the question of what actually caused the cessation of pathogen growth during the HR and what was the significance of the cell death part of the response.

The 1990s saw the beginning of what, in my opinion, has been an incredibly illuminating period in the field of plant–pathogen interactions, when hard data from molecular genetics began to replace the circumstantial evidence and hypothetical models prevalent in the previous decades. Hope for increased understanding of the HR came with the initial cloning of 'resistance (*R*) genes' that govern the expression of the HR during cultivar resistance in host plant species (Dangl, 1995) and the use of *Arabidopsis thaliana* mutants to reveal the existence of different defence signalling pathways, of which the salicylic acid-dependent pathway seemed most commonly associated with *R* gene-mediated resistance and the HR (Glazebrook, 2005). *Arabidopsis* mutants clearly demonstrated that different signalling pathways were differentially induced by different microbial pathogens (Thomma *et al.*, 2001) and that the *Arabidopsis* phytoalexin camalexin did, indeed, detrimentally influence pathogen growth *in planta*. However, the phytoalexin did not affect all pathogens tested and may not act alone (Thomma *et al.*, 1999). Interestingly, of those tested, the only pathogens affected by the phytoalexin were necrotrophs, which were not triggering an HR and for which there are no known gene-for-gene relationships with the plant (Glazebrook, 2005).

With such studies came the first widespread acceptance that the details of each plant–microbe interaction are likely to be unique with respect to the plant responses that are elicited and effects they have on the pathogen – a

conclusion that had been argued before on theoretical grounds (Heath, 1981a) and that has been further strengthened by subsequent molecular data (Jones and Dangl, 2006). Nevertheless, the current paucity of plant mutants with defects in the expression of specific defences means that there are still few, if any, examples of the HR where the factor(s) causing cessation of pathogen growth is unequivocally known.

Interest in the actual process of HR-associated cell death blossomed about a decade ago when a 'hot topic' in mammalian research was a form of programmed cell death (PCD) known as *apoptosis*. In the search for analogous processes in plants, it was realised that hypersensitive cell death, long known to be an active process requiring plant metabolism (Tomiyama, 1971), was a likely candidate. This idea was strengthened by the demonstration of a hallmark of apoptosis, the cleavage of plant nuclear DNA into oligonucleosomal fragments, during some examples of the HR (e.g. Ryerson and Heath, 1996). Since the constant association of defensive compounds with the HR makes it difficult to investigate the actual death process in isolation from defence responses, some of the more illuminating investigations of hypersensitive cell death have involved cytological studies using regular light microscopy (e.g. Tomiyama, 1971) and, more recently, computer-enhanced light microscopy and cytochemical techniques applicable to living cells. These techniques, often coupled with the use of pharmacological agents, have revealed that hypersensitive cell death generally requires an intact actin cytoskeleton and ion fluxes, and often involves the generation of reactive oxygen species (ROS) (Heath, 2000a). The discovery of the importance of ROS in plant–pathogen interactions, as in animal defence systems, was arguably another milestone in our understanding of how plants respond to potential pathogens. In the mid-1990s, a variety of studies suggested that plant cells respond to mechanical perturbations (Yahraus *et al.*, 1995) and pathogens or pathogen products with an 'oxidative burst' (Baker and Orlandi, 1995) that may have a signalling role in plant disease resistance (Lamb and Dixon, 1997). A direct role for ROS in hypersensitive cell death is suggested by the fact that a mutation in an *Arabidopsis* gene that limits photo-oxidative damage causes the HR to spread *Peronospora parasitica* (Mateo *et al.*, 2004). However, cytochemical and pharmacological studies do not support a direct role of ROS in all examples of the HR, correlating with the fact that video microscopy of living cells during the death process shows very distinct differences between different plant–microbe interactions in the speed in which the cell dies and the manner with which the various cell components are dismantled (Christopher-Kozjan and Heath, 2003).

With the increased interest in searching for plant parallels with animal apoptosis came more studies on other forms of plant PCD. Unlike the HR, developmental PCD is not associated with the accumulation of defence compounds and seems to have different cytological events accompanying cell death (Heath, 1998). If it is correct that cells are dismantled in different ways in different HRs and that these processes differ from those of other types of

plant PDCs, this adds yet another level of complexity to the HR and to the phenomenon of PCD in flowering plants.

Despite all we know about the HR, we have yet to answer the fundamental question of why cell death is needed to resist attack by cellular pathogens. The question is even more pertinent now that there are examples of experimental separation of this death from defence gene induction and disease resistance (Heath, 2000a) as well as data to suggest that hypersensitive cell death and defence gene activation during the HR may involve separate signalling pathways (Zhou *et al.*, 1998). One possibility worth exploring is that the primary role of cell death in the HR is to generate signals that trigger defence responses in local and distant non-infected tissue (Heath, 2000a).

1.3 The gene-for-gene hypothesis

The gene-for-gene hypothesis was proposed by Flor as a result of his studies, in the first half of the twentieth century, on the inheritance of pathogenicity in different races of the flax rust fungus, *Melampsora lini*, to cultivars of flax differing in genes for resistance. He suggested that 'for each gene that conditions reaction in the host there is a corresponding gene in the parasite that conditions pathogenicity' (Flor, 1971). With subsequent demonstrations that resistance in the plant and avirulence in the pathogen are usually dominant, the gene-for-gene hypothesis morphed into implying that for every gene for resistance in the host there is a corresponding gene for avirulence in the pathogen. In my opinion, few other concepts in plant disease resistance have had such a fundamental effect on the field of plant pathology and few have changed so dramatically with the advent of molecular genetics.

As well as being of enormous practical significance to plant breeders, the gene-for-gene hypothesis has dominated research into the basis of plant disease resistance, despite the fact that the number of systems clearly demonstrated to have gene-for-gene relationships is relatively small and these are predominantly diseases caused by pathogens that require living plant cells for their survival. The comparative ease of working with resistance that was genetically easily manipulated and that could be clearly distinguished from susceptibility by the common presence of the HR attracted researchers away from more genetically complex disease systems that showed only quantitative differences between resistance and susceptibility and involved preformed as well as inducible defences (see examples in Heath, 2000c).

The initial explanation for gene-for-gene relationships at the physiological level was that the gene for avirulence in the pathogen coded for an elicitor that directly interacted with the product of the corresponding gene for resistance in the plant, somehow causing an HR. However, trying to reconcile the available genetic data with the available knowledge of the regulation of gene expression and with the plethora of defence responses known to

accompany the HR proved problematic. Indeed, Ellingboe (1982) argued that failure to find ratios of avirulence (*Avr*) genes in the pathogen to *R* genes in the host other than 1:1 eliminated a role for phytoalexins in resistance since they were the end products of complex biochemical pathways controlled by many genes. However, he rightly advocated the need to identify the products of *Avr* and *R* genes as well as the use of mutants to provide proof of the role of specific defences in resistance.

The search in the 1970s for pathogen molecules that triggered phytoalexin production in a race-specific manner, as might be expected of an *Avr* gene product, had some success (Keen, 1975). However, most tested pathogen products were 'non-specific elicitors' that triggered responses in both resistant and susceptible plants, instead of being 'specific elicitors' that would trigger responses only in plants with specific *R* genes. It was not until 1984 that the first bacterial *Avr* gene was cloned (Staskawicz *et al.*, 1984) and some years later before one was cloned from a fungus (van Kan *et al.*, 1991) or a virus (Culver and Dawson, 1991). As more *Avr* genes were cloned, it became clear that *Avr* genes from different fungi or bacteria have little homology and some appear to be involved in pathogenicity, in accordance with the earlier argument by Person and Mayo (1974) that these genes had other functions prior to their products being co-opted as resistance-inducing recognition factors. However, even by the end of the twentieth century, relatively few specific elicitor molecules had been characterised, and not all had been proven to be products of *Avr* genes (Heath, 2000a) although most, but not all (Ji *et al.*, 1998), were proteins or peptides. For bacterial pathogens, we now know that the difficulty in isolating specific elicitors was due to the fact that bacterial *Avr* proteins generally are not secreted in culture but are delivered directly into the plant cell (Dangl and Jones, 2001).

The first *R* gene product to be identified was an anomaly in that it coded for an enzyme that degraded the host-selective toxin that acted as a pathogenicity factor for the nectrotrophic fungus, *Cochliobolus carbonum* race 1 (Meeley *et al.*, 1992). This pathosystem does not follow the normal pattern for those involving host-selective toxins, whereby pathogenicity in the pathogen is dominant and resistance in the plant is recessive. Thus, it acts as a reminder that much of our information, even today, applies to a limited number of experimentally manipulatable pathosystems, and there are probably exceptions to every rule.

Once a more typical *R* gene was cloned (Martin *et al.*, 1993), cloning of others progressed rapidly, many from *Arabidopsis* (Dangl and Jones, 2001). By 2001, five classes of *R* genes were recognised and there were data to show that, depending on the pathosystem, *Avr* gene products may or may not bind directly to *R* gene products (Dangl and Jones, 2001). With the concurrent demonstration that *R* gene activation results in the deployment of signalling pathways that lead to the expression of PR proteins, it finally became clear that there are more plant genes involved in 'gene-for-gene' resistance than the *R* gene itself.

It could be argued that for more than 20 years, 'tunnel vision' among researchers due to their focus on the apparent gene-for-gene relationship governing resistance of specific genotypes of host plants to specific genotypes of the pathogen had been a major obstacle in reconciling genetic and physiological information on disease resistance within host species. It is now apparent that the reason why classical genetic studies did not reveal the myriad of genes now known to be involved in the expression of *R* gene-controlled resistance is a lack of natural variation in these genes within the plant population and/or a degree of redundancy within defensive responses. The revelation of the true complexity of gene-for-gene interactions had to await the development, in other disciplines, of techniques used in modern molecular genetics and the discovery of the incredible usefulness of the weed plant, *Arabidopsis*.

1.4 Host versus non-host resistance

When I first started working on non-host resistance in the 1970s, the term was not in common usage. Many physiological or cytological studies of disease resistance did not make clear distinctions between the gene-for-gene resistance seen in host species towards different genotypes of a specific pathogen, the resistance of an otherwise susceptible plant species to forms of its pathogen that are adapted to other plants or 'true' non-host resistance in which all genotypes of a plant species are resistant to all genotypes of the pathogen species. Several times I was told that studying non-host resistance was a waste of time because it could not be investigated by classical genetics, that this resistance was uninteresting because it was caused by passive physical or chemical barriers or that it was just a manifestation of the plant 'not being a suitable host' for the pathogen. However, within a decade it was more widely appreciated that non-host resistance was of considerable significance since every plant is a non-host to the majority of plant parasites it encounters. Moreover, the narrow host range of most pathogens implies that non-host resistance is difficult to overcome – a desirable feature when breeding for resistance. A suggested explanation for this durability of non-host resistance came from data indicating that this resistance was non-specific, multicomponent and often involved both preformed and inducible defences (Heath, 1981a,b), a conclusion that still seems viable today (Lipka *et al.*, 2005; Jones and Dangl, 2006).

In the mid-1970s, it was suggested from genetic considerations that the gene-for-gene resistance seen in interactions between a specific pathogen genotype and a specific host genotype must be superimposed on a 'basic compatibility' between these pathogens and their host plant species (Person and Mayo, 1974; Ellingboe, 1976). However, it was only with increased information on non-host resistance that it became apparent that this basic compatibility required each species of pathogen to evolve traits to specifically

overcome the non-host defences of its host (Heath, 1981a). For biotrophic fungal pathogens and some bacteria, we now know that these traits include the ability to specifically suppress certain defence responses by mechanisms that differ between pathosystems (Heath, 2002; Abramovitch and Martin, 2004; Glazebrook, 2005; Fujikawa *et al.*, 2006; Jones and Dangl, 2006).

With an increasing interest in non-host resistance came the debate on whether inducible components of this resistance were simply the result of multiple *Avr*–*R* gene interactions or were elicited by non-specific recognition events involving non-specific elicitors. Extensions of these questions were whether *R* genes were involved in host resistance that was not dependent on the pathogen's genotype (race–non-specific resistance) and whether *R* genes could be exploited in breeding for a disease resistance that was as durable as non-host resistance (Johnson, 1984). Within the last decade, this debate has resurfaced with the concept that non-host plants respond to potential pathogens via the perception of generic microbial products (non-specific elicitors) by transmembrane 'pattern recognition receptors' (Jones and Dangl, 2006), and the potentially contrary suggestion that there may be pathogen-specific recognition in some examples of non-host resistance in which a single pathogen molecule and/or single pathogen gene is all that needed for the elicitation of a defensive response (usually a visible HR) (Heath, 2001). These different data-based conceptions of non-host resistance, together with evidence that certain *R* genes may stimulate a basal (i.e. non-host type) defence pathway (Xiao *et al.*, 2005), suggest that there is considerable diversity in, and cross-talk between, the molecular events that occur in host or non-host disease resistance.

Despite an overlap in molecular events that may occur in non-host and host resistance, the former is commonly expressed earlier after pathogen contact than the latter. As a result, the HR may be pre-empted by earlier defence responses in some examples of non-host resistance to bacteria (e.g. Soylu *et al.*, 2005) and pre-penetration resistance to fungi (e.g. Mellersh *et al.*, 2002; Lipka *et al.*, 2005). However, if a fungus does manage to reach the cell lumen in a non-host, the cell usually dies. Interestingly, video microscopy of living cells coupled with pharmacological studies suggests that the process of this non-host hypersensitive cell death is not the same as that exhibited by the same plant when it is acting as a resistant host (Christopher-Kozjan and Heath, 2003). Also significant is evidence that there is some signalling commonality in the cell death triggered by a variety of *R* genes (Muskett *et al.*, 2002; Gabriels *et al.*, 2007). It is an intriguing idea that *R* genes commonly trigger a different hypersensitive cell death from that evolved to non-specifically defend plants against microbial invaders.

1.5 Future promise

During the last 40 years, we have learned an enormous amount about how plants defend themselves against pathogen attack. In addition to the

features discussed above, we now know that localised defence responses may prime the rest of the plant to exhibit increased resistance to further invasion (Kuć, 1982), a phenomenon originally called induced resistance, but now generally known as systemic acquired resistance (SAR). There is also some evidence that damaged plants communicate stress to their neighbours (Baldwin and Schultz, 1983), although such studies usually involve herbivory, rather than pathogen, damage. Encouragingly, some disease resistance concepts based on deductive reasoning have not changed over the years and have been strengthened by new data, but others have changed dramatically. Time has not changed my opinion that careful cytological studies provide important, fundamental and objective information on any plant–pathogen interaction, and should be the first line of investigation. There have been, for example, cases where simple cytological observations of the manner of fungal pathogen growth, and it is time of cessation in resistant plants, could have channelled biochemical studies into more relevant time frames and would have revealed flaws in disease resistance models. Another factor that is now better appreciated is that with fungal and oomycete pathogens in particular, critical events during the infection process may be localised to a single cell, making it important to be able to distinguish these events from others that may subsequently occur in adjacent non-penetrated neighbours. Studying single cells in plants is technically difficult, although several laboratories have been successful at looking at pathogen-triggered gene expression in individual epidermal cells (Matsuda *et al.*, 1997; Mould *et al.*, 2003; Gjetting *et al.*, 2007). Such studies coupled with cytological and cytochemical investigations are beginning to suggest that significant plant–pathogen interactions may begin moments after contact between the two organisms. For example, it seems that for interactions with some biotrophic fungi, whether the plant cell is going to respond as a non-host, a susceptible host or resistant host is determined by events that happen prior to the complete penetration of the plant cell wall (Heath, 2002; Mould *et al.*, 2003). Given that there have been considerable recent advances in imaging the cell biology of plant–microbe interactions (Heath, 2000b; Koh and Somerville, 2006) and there now seems to be a wider appreciation of the value of combining cytological studies with other types of investigations, it is likely that more attention will be paid in the future to the spatial organisation of recognition events and defence responses between and within affected cells (e.g. Robatzek, 2007).

If we have learned anything from the last 40 years of plant disease resistance research, it is that the plant's interaction with potential microbial invaders is much more complex and sophisticated than originally imagined. We now know that different pathogens have different modes of infection and that different plants have different defence biochemistries, thereby requiring each pathogen to evolve specific adaptations to overcome the specific mix of physical and biochemical features of its host. As the data from studies with *Arabidopsis* mutants have confirmed, this means that the exact details of each plant–pathogen interaction will be unique – even with respect

to resistance of the same plant to the same pathogen species governed by different genes for resistance (Eulgem *et al.*, 2004). Therefore, we still have a lot to learn when one considers that disease resistance studies have focused on relatively few pathosystems, given that there are over 250 000 extant species of flowering plants and an unknown, but probably huge, number of microbial plant pathogens. However, the application of molecular genetics has also revealed commonalities in terms of signalling systems and defence gene involvement, both between plants and between different types of resistance, implying that all types of plant resistance, be it non-host, host, age-related, SAR or organ-specific, likely exploit different parts and/or combinations of the same complex web of interacting signalling systems and defensive biochemicals and processes that each plant possesses. Therefore, the uniqueness of each interaction is based on which signalling pathways are triggered or suppressed by the pathogen, the sensitivity of the pathogen to each defensive product or process and variation between plants (and/or plant parts) in whether certain defence gene expression is constitutive or has to be induced (Heath, 2000c, 2001). Attempts to fully understand even a few examples of disease resistance seem likely to keep researchers occupied for some time to come.

The recent exponential increase in our understanding of plant–microbe interactions has primarily been the result of the application of molecular genetics to pathogen interactions with *Arabidopsis*, and the result of looking for homologues of *Arabidopsis* genes in other plants (e.g. Pajerowska *et al.*, 2005). The surge in interest in non-host resistance is, in large part, due to the fact that genes involved in this process now can be revealed without having to resort to classical genetic studies involving the crossing of unrelated species. The ability to use genome arrays to monitor the expression profiles of huge numbers of genes after infection (e.g. Eulgem *et al.*, 2004) has removed much of the bias that a researcher might have in choice of plant response to examine, and has served to emphasise dramatically how responsive plants are to pathogen attack. As genome arrays for more plant species become available, other pathosystems can be examined in similar detail. With all this increased information comes the challenge of designing computational methods to manage the data and to sort what is important in disease resistance from what is secondary. For this, gene silencing and other techniques to selectively prevent or stimulate gene expression will be important. However, it is currently easier to identify the genes that are involved in disease resistance than it is to identify what happens in the plant cell after the expression of these genes, particularly when it comes to the trafficking of molecules between cellular compartments and the spatial and biochemical events that actually stop pathogen growth. I hope that this is where there will be great strides forward in the future.

For most of the last 40 years, there have been only sporadic attempts at comparing disease resistance in animals and plants, but this has changed in the last decade or so with the ability to compare genes, proteins and

signalling systems across kingdoms. As a result, emphasis has shifted to common underlying principles between animal and plant defence systems rather than their differences. In part, this explains some of the terminology changes that have recently taken place. For example, the 'basic resistance' (Heath, 1981a) in each plant that accounts for non-host resistance and for 'residual' disease resistance in susceptible plants has given rise to the term 'basal resistance' (Jones and Dangl, 2006) or 'basal immunity' (Robatzek, 2007), and non-specific plant defences are now considered part of the plant's 'innate immunity' to disease, and there are reviews on the 'plant immune system' (Jones and Dangl, 2006). At the level of receptors and signalling cascades, conservation across kingdoms of eukaryotic organisms is not unexpected, but this unifying approach can be problematic if it narrows conceptual thinking. I have already seen a change in the study of hypersensitive cell death where researchers either force their observations into what they expect to see on the basis of the process of mammalian apoptosis or channel their studies into looking for specific apoptotic features. Animal pathosystems may well provide some clues as to the nature of some plant–pathogen interactions, but we should also appreciate that plants have been genetically isolated from animals and subject to different evolutionary pressures, for over 400 million years. Even within the plant kingdom, although some genes involved in defence signalling appear to be conserved between higher plant species (e.g. Muskett *et al.*, 2002), others may have changed with time in role and function (Mcdowell and Simon, 2006). Particularly intriguing are data suggesting that plant genes involved in disease resistance also have a role in plant development (e.g. Holt *et al.*, 2002). We are just at the beginning of an exciting period where molecular tools and innovative classical genetic systems (Jafary *et al.*, 2006) are now available to determine just how plant disease resistance and pathogen pathogenicity have evolved during the evolution of higher plants.

As demonstrated by the other chapters in this volume, the distance that we have travelled from Wood's all-encompassing book in 1967 is astounding. One disappointing feature, however, is that there are very few examples where our increased knowledge of the basis of plant disease resistance has resulted in new ways of controlling plant diseases in the field that are commercially viable and in widespread use. The overexpression of defence response genes in transgenic plants has shown promise for some pathogens (e.g. Mackintosh *et al.*, 2007), but most successful has been the use of pathogen infection or other treatments to induce systemic plant resistance, although the level of disease control is not always sufficiently high for the treatments to be used alone (Walters *et al.*, 2005). It seems certain that the next 40 years will bring as many, or more, revelations on the basis of plant disease resistance as did the last, and it is to be hoped that our basic understanding of plant–microbe interactions at the molecular and cellular level can eventually be exploited to produce a significant effect on the disease resistance of our domesticated plants.

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Chapter 2

PATHOGEN-ASSOCIATED MOLECULAR PATTERNS (PAMP) AND PAMP-TRIGGERED IMMUNITY

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Abstract: Recent years have witnessed significant advances in our understanding of the molecular mechanisms that govern plant disease resistance. One of the most important benefits of this development is a new concept of how seemingly unrelated types of microbial resistance in plants are functionally linked and how they might be evolutionarily related. For a long time, research on microbial elicitor-induced plant non-cultivar-specific defences and microbial avirulence factor-induced host plant cultivar-specific defences had coexisted without providing an integrated model for plant disease resistance. Recent landmark insights have significantly reshaped our view of plant immunity. Among those achievements are (i) the realisation of conceptual and mechanistic similarities in animal and plant immunity, (ii) the identification of plant pattern recognition receptors that recognise pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs), (iii) evidence that PAMP-triggered immunity is a biologically important element of the plant immune system and (iv) that suppression of PAMP-triggered immunity is a major strategy of adapted pathogens to facilitate infections of susceptible host plants. This chapter highlights recent progress made in PAMP research, with particular emphasis on the findings mentioned above.

Keywords: pathogen-associated molecular pattern; pattern recognition; receptor, immunity; plant defence; effector

2.1 The concept of plant immunity

Numerous recent papers addressing diverse aspects of plant defence, disease resistance or susceptibility have adopted a terminology that differs largely

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from that applied 5 years ago. This development is characterised by the use of such terms as innate immunity, pathogen-associated molecular pattern (PAMP), pattern recognition receptors (PRRs), effectors and so on. An immunity-associated terminology has in large parts replaced a more traditional phytopathological vocabulary that dominated the literature for many years. As there is still some confusion among readers in the field, there is a need to address the adequacy of an immunity-associated terminology in describing plant disease resistance. In general, the term 'immunity' refers to the state of having sufficient biological defences to avoid infection, disease or other unwanted biological invasion. As this definition applies to all multicellular eukaryotic systems, it is appropriate to describe the ability of plants to cope with microbial infections as an 'immune' response. The question why such a change in vocabulary has occurred only recently is pertinent. Notwithstanding early discussions of analogies between plant disease resistance genes and the major histocompatibility complex of animal systems (Dangl, 1992), the driving force for this shift has been the recent re-evaluation of innate immunity in jawed vertebrates as a prerequisite for the containment of microbial infection and for mobilisation of the adaptive immune system. Numerous pioneering papers have since highlighted striking similarities in the molecular organisation of non-self-recognition and antimicrobial defence systems in animals and plants (Nürnberger *et al.*, 2004; Ausubel, 2005; Chisholm *et al.*, 2006; Jones and Dangl, 2006). These similarities encompass the nature of microbial patterns recognised by innate immune systems in both lineages and extend to the molecular architecture of pattern recognition complexes and the production of antimicrobial products that eventually halt microbial infections. In the light of these findings, the adoption of an immunity-associated terminology by molecular plant pathologists appears to be appropriate. Related terminologies in plant and animal immunity should, however, be used with caution since significant differences between the systems are clear. For example, the term 'innate' in conjunction with plant immunity appears to be superfluous as all immunity-associated traits of plants are inheritable. In animal systems, this term is useful as it discriminates germ line-encoded innate immunity from adaptive immunity that is characterised by genomic recombination of antigen receptors and clonal expansion of particular lymphocyte populations (Medzhitov, 2007). Adaptive immunity does not exist in plants and therefore the term 'innate' in conjunction with plant immunity could be misinterpreted.

Plants provide various habitats that can be invaded by microorganisms, including the root, leaves, flowers and vascular system. Some of these niches (for example the root) are colonised constitutively by an endogenous microflora. The impact of microbial colonisation on host fitness critically depends on the microbial adaptation strategy. The establishment of various types of symbiosis represents a case in which the impact of infection is a positive one. The relationship between two living organisms where one benefits and the other is not significantly harmed or helped has been described in

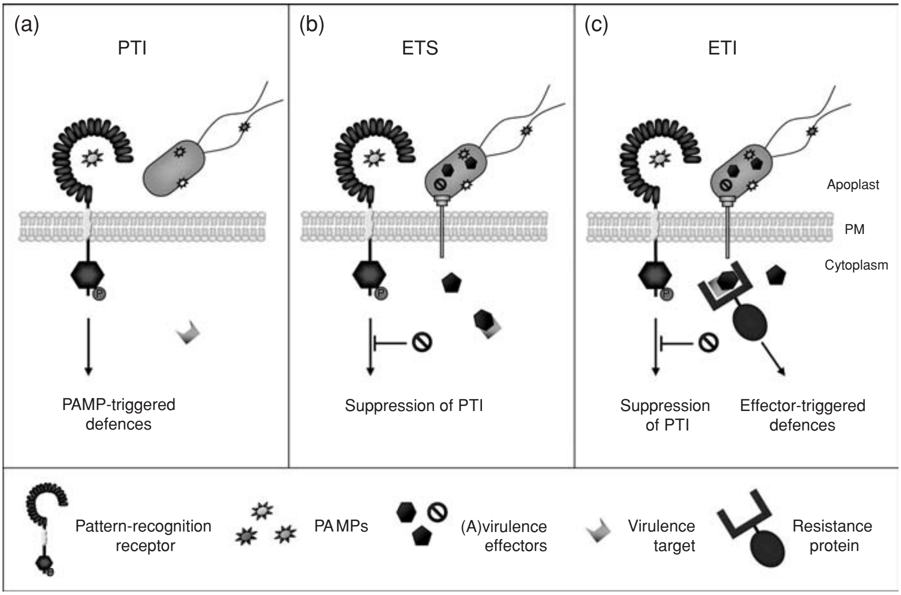

Figure 2.1 The plant immune system – an evolutionary view. (a) Pattern recognition receptor-mediated perception of microbial PAMPs triggers immune defences (PTI) that restrict growth of adapted pathogens (basal immunity) and halt growth of non-adapted pathogens (species or non-host immunity). (b) Virulent pathogens have acquired effectors that are translocated into plant cells and that facilitate infections (effector-triggered susceptibility, ETS). Some of these effectors suppress PTI. (c) Resistance protein-mediated effector recognition gives rise to effector-triggered immunity (ETI) that halts growth of adapted pathogens.

plant–microbe associations. The term *commensalism* commonly used in animal ecology to refer to this phenomenon is, however, unusual in the plant literature. In other cases, microbial colonisation can be disadvantageous to the host and such microbes are referred to as pathogens. The adaptation of infectious pathogens to a specific environment in the host depends on the activity of virulence factors that enable host ingress, evasion of host defences, microbial nutrition and proliferation. In turn, microbial infections are assumed to be the driving force in the evolution of plant immunity.

In brief, the plant immune system consists of two evolutionarily linked branches (Fig. 2.1). The primary plant immune response is referred to as PAMP-triggered immunity (PTI) and has evolved to recognise invariant structures of microbial surfaces termed pathogen-associated molecular patterns (PAMPs) (Nürnberger *et al.*, 2004; Ausubel, 2005; Zipfel and Felix, 2005; Chisholm *et al.*, 2006; Jones and Dangl, 2006). PAMP-induced immune responses are important for immunity to microbial infection of whole plant species (species or non-host immunity) and for basal immunity in susceptible host plant cultivars (Nürnberger and Lipka, 2005; Bittel and Robatzek, 2007). Subversion of PTI by microbial effectors (effector-triggered susceptibility,

ETS) is believed to be one of the key strategies of successful pathogens to grow and multiply on host plants (Alfano and Collmer, 2004; Chisholm *et al.*, 2006; Jones and Dangl, 2006). In the co-evolution of host–microbe interactions, individual plant cultivars have acquired resistance (R) proteins that guard microbial effector-mediated perturbations of host cell functions and thereby trigger plant immune responses. This type of plant defence is referred to as effector-triggered immunity (ETI) and is synonymous to pathogen race/host plant cultivar-specific plant disease resistance (Ausubel, 2005; Chisholm *et al.*, 2006; Jones and Dangl, 2006). In the following sections, we review in detail the current knowledge of PTI. Readers interested in the molecular basis of ETI are referred to Chapters 3 and 7 of this volume.

2.2 Pathogen-associated molecular patterns

Immune recognition is a key prerequisite for the activation of inducible host defences. It is based on the detection of molecular structures (or 'patterns') that are unique to microorganisms and thereby enable the host to discriminate between host-derived self and microbial non-self (Akira *et al.*, 2006; Ferrandon *et al.*, 2007; Medzhitov, 2007). In 1997, Medzhitov and Janeway provided a terminology to describe the elements and processes implicated in innate immunity in various animal systems (Medzhitov and Janeway, 1997). These authors coined the term PAMPs for triggers of immune responses in organisms as diverse as humans, mice, crustaceans or insects. Immune defences evoked in jawed vertebrates include the pro-inflammatory cytokine and chemokine production that mediate inflammatory responses as well as the production and secretion of antimicrobial, proteinaceous defensins (Akira *et al.*, 2006; Medzhitov, 2007). Likewise, PAMP-induced synthesis of a blend of antimicrobial peptides is a hallmark of innate immunity in *Drosophila melanogaster* (Girardin *et al.*, 2002; Ferrandon *et al.*, 2007). PAMPs that trigger innate immune responses in various vertebrate and non-vertebrate organisms include lipopolysaccharides (LPS) from gram-negative bacteria, peptidoglycans from gram-positive bacteria, eubacterial flagellin, methylated bacterial DNA fragments and fungal cell wall-derived glucans, chitins, mannans and proteins (Aderem and Ulevitch, 2000; Girardin *et al.*, 2002; Ferrandon *et al.*, 2007; Medzhitov, 2007). Medzhitov and Janeway's terminology has had an enormous impact on molecular plant pathology mainly because many of the pathogen-associated patterns with immunity-modulating features had long been known to be (general) elicitors of cultivar non-specific defences in many plants (Boller, 1995; Vorwerk *et al.*, 2004; Zipfel and Felix, 2005) (Table 2.1). For example, structural elements of LPS from gram-negative bacteria, an N-terminal 22-mer fragment of eubacterial flagellin (flg22) or peptidoglycans derived from gram-positive and gram-negative bacteria, are potent inducers of defence-associated responses in various plant species (Felix *et al.*, 1999; Newman *et al.*, 2002; Gust *et al.*, 2007). This new insight suggested a common evolutionary concept for pattern recognition and activation of antimicrobial

Table 2.1 Selected microbial PAMPs and host-derived DAMPs that trigger PTI in plants

PAMP	Origin	Minimal structural motif required for defence activation	Sensitive plants	Reference
Lipopoly-saccharides	Gram-negative bacteria (xanthomonads, pseudomonads)	Lipid A and O-antigen oligosaccharides	Pepper, tobacco, *Arabidopsis*	Meyer *et al.* (2001), Newman *et al.* (2002), Zeidler *et al.* (2004) and Bedini *et al*. (2005)
Flagellin	Gram-negative bacteria	flg22 (amino-terminal fragment of flagellin)	Tomato, *Arabidopsis*	Felix *et al.* (1999)
Elongation factor (EF-Tu)	Gram-negative bacteria	elf18 (N-acetylated amino-terminal fragment of EF-Tu)	*Arabidopsis* and other Brassicaceae	Kunze *et al.* (2004)
Harpin	Gram-negative bacteria (pseudomonads, erwinia)	Undefined	Cucumber, tobacco, tomato, *Arabidopsis*	Wei *et al.* (1992), He *et al.* (1993) and Lee *et al.* (2001a)
Cold shock protein	Gram-negative bacteria Gram-positive bacteria	RNP-1 motif (amino-terminal fragment of the cold shock protein)	Solanaceae	Felix and Boller (2003)
Necrosis-inducing proteins	Bacteria (*Bacillus* spp.), fungi (*Fusarium* spp.), oomycetes (*Phytophthora* spp., *Pythium* spp.)	Undefined	Dicotyledonous plants	Bailey (1995), Veit *et al.* (2001), Fellbrich *et al.* (2002), Qutob *et al.* (2002), Mattinen *et al.* (2004) and Pemberton and Salmond (2004)
Transglu-taminase	Oomycetes (*Phytophthora* spp.)	Pep-13 motif (surface-exposed epitope of the transglutaminase)	Parsley, potato, grapevine, tobacco, *Nicotiana benthamiana*	Nürnberger *et al.* (1994) and Brunner *et al.* (2002)
Lipid-transfer proteins (elicitins)	Oomycetes (*Phytophthora* spp., *Pythium* spp.)	Undefined	Tobacco, turnip	Osman *et al.* (2001)

Table 2.1 (*Continued*)

PAMP	Origin	Minimal structural motif required for defence activation	Sensitive plants	Reference
Xylanase	Fungi (*Trichoderma* spp.)	TKLGE pentapeptide (surface-exposed epitope of the xylanase)	Tobacco, tomato	Enkerli *et al.* (1999) and Ron and Avni (2004)
Invertase	Yeast	N-Mannosylated peptide (fragment of the invertase)	Tomato	Basse *et al.* (1993)
β-Glucans	Fungi (*Pyricularia oryzae*) Oomycetes (*Phytophthora* spp.) Brown algae	Tetraglucosyl glucitol Branched hepta-β-glucoside Linear oligo-β-glucosides	Tobacco, rice, *Fabaceae*	Klarzynski *et al.* (2000), Yamaguchi *et al.* (2000) and Fliegmann *et al.* (2004)
Sulfated fucans	Brown algae	Fucan oligosaccharide	Tobacco	Klarzynski *et al.* (2003)
Chitin	All fungi	Chitin oligosaccharides (degree of polymerisation > 3)	Tomato, *Arabidopsis*, rice, wheat, barley	Baureithel *et al.* (1994) and Ito *et al.* (1997)
Ergosterol	All fungi	19	Tomato	Granado *et al.* (1995)
Cerebrosides A, C	Fungi (*Magnaporthe* spp.)	Sphingoid base	Rice	Koga *et al.* (1998)
Oligouronides	Plant cell wall pectins	Oligomers	Tobacco, *Arabidopsis*	Darvill *et al.* (1994)
Cellodextrins	Plant cell wall cellulose	Oligomers	Grapevine	Aziz *et al.* (2007)
Cutin monomers	Plant cuticle	Dodecan-1-ol	Cucumber, tomato, apple	Fauth *et al.* (1998)

counter-defence in multicellular eukaryotes and highlighted the importance of elicitor recognition in plant immunity. This had been long awaited because evidence for a causal link between elicitor-induced plant responses and plant disease resistance was still lacking.

The term PAMP is really a misnomer because such structures are not only present on pathogenic microbes, but also found in non-pathogenic microorganisms (Ausubel, 2005). There have been attempts in the recent (plant,

but not animal) literature to introduce more precise terms such as MAMP (microbe-associated molecular pattern) (Ausubel, 2005) or MIMP (microbe-induced molecular pattern) (Mackey and McFall, 2006). While these terms have their merits, we suggest keeping with the 'historical' term PAMP for the simple reason of maintaining an understanding among researchers in animal and plant immunity.

PAMPs are abundant, conserved structures (patterns) that are typical of whole classes of pathogens (Medzhitov and Janeway, 1997). Such patterns appear to be absent in eukaryotic host organisms but are indispensable for the microbial lifestyle. For these reasons, PAMPs are ideal determinants for host-encoded non-self recognition systems. PAMP-like elicitors of plant defences match these characteristics (Brunner *et al.*, 2002; Felix and Boller, 2003). For example, Pep-13 (Nürnberger *et al.*, 1994), a surface-exposed peptide motif of a *Phytophthora* cell wall transglutaminase (TGase) (Brunner *et al.*, 2002), likely serves as a recognition determinant for the activation of defence in various plants, including parsley, potato, grapevine and *Nicotiana benthamiana* (*Nb*) (Halim *et al.*, 2005; H.-H. Kassemeyer and T. Romeis, personal communication) in response to attempted infection by various *Phytophthora* species. Pep-13 sequences are conserved among *Phytophthora* TGases, but are not found in plant proteins. The Pep-13 motif is essential for elicitor activity and for TGase activity of the protein (Brunner *et al.*, 2002). Moreover, individual TGase isoforms containing the Pep-13 motif are expressed at all stages of the life cycle of *Phytophthora infestans*, including plant infection, suggesting a crucial role of these enzymes in *Phytophthora* biology (Fabritius and Judelson, 2003). Likewise, a cold shock-inducible RNA-binding protein from various gram-positive bacteria (RNP-1) that induced innate immune responses in tobacco was shown to meet the characteristics of a PAMP (Felix and Boller, 2003). A central peptide (csp22) within RNP-1 was defined that is conserved among all bacterial RNP-1 orthologues analysed. This region is not only indispensable for the RNA-binding activity of the protein but also necessary and sufficient for its defence-inducing capacity. Other microbe-specific structures, such as fungal chitin, oomycete glucans, bacterial flagellin and the bacterial elongation factor EF-Tu (Kunze *et al.*, 2004), are predicted to be indispensable for the microbial host and are thus equivalent to PAMPs triggering innate immunity in animal systems (Medzhitov and Janeway, 1997).

Plant cells encounter a variety of these signals when interacting with microorganisms in vivo. A comprehensive list of PAMPs with known immunity-stimulating activities in plants is given in Table 2.1. It is envisaged that the activation of inducible plant defences results from the recognition of complex patterns that build the microbial surface (Nürnberger and Lipka, 2005; Zipfel and Felix, 2005). For example, the cell walls of many phytopathogenic fungi harbour chitins, N-mannosylated glycopeptides and ergosterol, all of which have been reported to trigger plant defence responses (Basse *et al.*, 1993; Baureithel *et al.*, 1994; Granado *et al.*, 1995). Various phytopathogenic gram-negative bacteria harbour plant defence-stimulating LPS and flagellin and produce HrpZ (harpins; bacterial effector proteins that may function

as pathogenicity factors during bacterial infection of plants and that are delivered into the plant apoplast via the bacterial type III secretion system) upon contact with plants (Wei *et al.*, 1992; He *et al.*, 1993; Felix *et al.*, 1999; Lee *et al.*, 2001a; Newman *et al.*, 2002). Moreover, phytopathogenic oomycetes of the genera *Phytophthora* and *Pythium* possess defence-eliciting heptaglucan structures, elicitins and other cell wall proteins (Mithöfer *et al.*, 2000; Kamoun, 2001; Veit *et al.*, 2001; Fellbrich *et al.*, 2002; Qutob *et al.*, 2002). Although not all plant species may recognise and respond to all these signals, plant cells have recognition systems for multiple patterns derived from individual microbial species. This is exemplified by tobacco and *Arabidopsis* cells which recognise *Pseudomonas syringae*-derived harpins and flagellin (Desikan *et al.*, 1999; Felix *et al.*, 1999), while tomato cells perceive fungal chitin fragments, glycopeptides and ergosterol (Boller, 1995). Altogether complex pattern recognition in plants is another phenomenon that resembles the activation of innate defence in animals. For example, innate immune responses in humans are activated by gram-negative bacteria-derived LPS, flagellin and unmethylated CpG dinucleotides, which are characteristics of bacterial DNA (Aderem and Ulevitch, 2000; Akira *et al.*, 2006; Medzhitov, 2007). It is currently unclear whether recognition of multiple signals derived from one pathogen can mediate more sensitive perception or whether redundant recognition processes act as independent backup systems in the same or different tissues. Peptidoglycans from gram-positive bacteria acted synergistically on inflammatory cytokine production in human mononuclear macrophages when added simultaneously with gram-negative bacteria-derived LPS (Wolfert *et al.*, 2002). Likewise, concomitant addition of *P. syringae*-derived LPS and HrpZ1 resulted in synergistic activation of antimicrobial phytoalexin production in parsley cells (our unpublished data). In contrast, bacterial flagellin and EF-Tu appear to activate a common set of signalling events and defence responses, but with additive rather than synergistic effects (Zipfel *et al.*, 2006). In either case, many eukaryotic hosts take advantage of concomitant recognition of microbial patterns.

Recently, PAMPs have been defined as triggers of numerous plant immune responses except apoptotic-like, programmed cell death (hypersensitive response, HR), which is in contrast to ETI responses in resistant host plant cultivars that frequently involve HR (Jones and Dangl, 2006). However, some microbe-derived PAMPs were shown to trigger HR cell death in a plant cultivar non-specific manner. For example, a pentapeptide motif found within fungal cell wall-associated xylanases was sufficient for the HR elicitor activity of the intact protein (Rotblat *et al.*, 2002). Moreover, flagellin preparations from various, but not all, *Pseudomonas* species exhibited cell-death-inducing activities in tobacco or rice (Che *et al.*, 2000; Hann and Rathjen, 2007) but not *Arabidopsis*, whereas Pep-13 triggered salicylic acid (SA)-dependent cell death in potato but not parsley (Halim *et al.*, 2005). Thus, some PAMPs trigger HR in a species-specific manner.

PAMPs are commonly considered constitutive building blocks of microbial surfaces. However, phytopathogenic gram-negative bacterial species of

the genera *Pseudomonas*, *Erwinia* or *Ralstonia* express HrpZ, HrpN or PopA ('harpins') proteins that are massively produced but secreted only upon attempted microbial infection of plants (Alfano and Collmer, 2004). Although secreted in a type III secretion system (TTSS)-dependent manner, 'harpins' are not translocated into plant host cells like typical TTSS effectors but appear to be targeted to the apoplastic plant–bacteria interface. Harpins have been shown to contribute to microbial virulence (Bauer *et al.*, 1995) and to form ion-conducting pores in synthetic and plant lipid bilayer systems (Lee *et al.*, 2001b; Racape *et al.*, 2005). This suggested that these proteins facilitate nutrient delivery into the apoplastic space or effector delivery into host cells as part of the TTSS effector translocon. Very recently, HrpZ has been assigned a function in effector delivery in *P. syringae*, thus rendering the latter assumption more likely (Kvitko *et al.*, 2007). Remarkably, all 'harpins' trigger cultivar non-specific plant immunity including HR cell death in various plant species (Lee *et al.*, 2001a; Alfano and Collmer, 2004). A detailed structure–function analysis of the HrpZ protein from *P. syringae* pv. *phaseolicola* revealed that the full-length protein was required for its virulence-associated pore-forming activity, while a C-terminal portion of the protein was sufficient for activation of plant immune responses. Since the C-terminal portion was also sufficient to bind to a previously identified HrpZ-binding site in tobacco and parsley membranes (Lee *et al.*, 2001a), we assume that HrpZ plays dual roles in plant–microbe interactions as virulence-promoting factor and as trigger of PTI (our unpublished data).

2.3 Damage-associated molecular patterns

In addition to microbial PAMPs or ETI-inducing effectors, breakdown products of the plant cell wall ('endogenous elicitors') are known to elicit plant immune responses (Vorwerk *et al.*, 2004). For example, plant cell wall-derived oligogalacturonide fragments (pectic fragments derived from primary plant cell walls), cellulose fragments (cellodextrins) or cutin monomers stimulate plant immune responses that are indistinguishable from those triggered by microbe-derived PAMPs (Darvill *et al.*, 1994; Fauth *et al.*, 1998; Aziz *et al.*, 2007). Such plant-derived elicitors are likely released by glucohydrolytic activities from attacking microbes and are reminiscent of animal tissue-derived 'danger' or 'alarm' signals. These animal host-derived patterns are produced upon microbial infection, mechanical injury or necrotic cell death (called damage-associated molecular patterns, DAMPs); function as sensors of host damage or cellular distress; and activate innate immune responses (Gallucci and Matzinger, 2001; Matzinger, 2002; Seong and Matzinger, 2004). There is a growing list of animal host-derived immunostimulators, including hyaluronan, heat shock proteins (Hsp), glucose starvation proteins, fibronectins, β-defensins and cardiolipin, many of which are recognised by PRRs that sense 'classical' PAMPs (human TLR4,

for example, recognises bacterial LPS, host-derived Hsp70 and breakdown products of host hyaluronan) (Matzinger, 2007). All these signals have a feature in common in that they are not released in intact and healthy tissues and are therefore well suited as determinants of damaged or non-intact host-self. Because these endogenous immunostimulators are difficult to fit into self/non-self-discrimination models (referred to as the 'stranger' model), it has been proposed that animal innate immune cells may rather recognise 'danger' signals that comprise both non-self-representing microbial PAMPs and damaged self-representing DAMPs ('danger' model) (Matzinger, 2002). To our understanding, activation of plant immunity by pectin, cellulose or cutin fragments constitutes a phenomenon that is conceptually similar to DAMP-induced animal immunity.

2.4 Pathogen-derived toxins

Microbial toxin-induced plant immunity is a paradoxical phenomenon that has been known for a long time but is little understood. Phytopathogenic microorganisms produce numerous cytolytic toxins that function as major virulence determinants (Glazebrook, 2005; van't Slot and Knogge, 2002). Phytopathogenic necrotrophic fungi, for example, synthesise host selective and host non-selective compounds that facilitate killing of host plant tissue (van't Slot and Knogge, 2002; Wolpert *et al.*, 2002; Gijzen and Nürnberger, 2006; Qutob *et al.*, 2006). An intriguing characteristic of many of these mycotoxins is that they not only harm but also trigger individual facets of the plant defensive arsenal. For example, certain *Fusarium* spp. produce the sphinganine toxin fumonisin B1 (FB1) that elicits cytolysis of plant and animal cells probably through competitive inhibition of ceramide synthase, an enzyme involved in sphingolipid biosynthesis (Wang *et al.*, 1996; Tolleson *et al.*, 1999). In addition to cell death, FB1 triggers accumulation of reactive oxygen species (ROS), deposition of callose, defence-related gene expression and production of the phytoalexin camalexin in *Arabidopsis* (Asai *et al.*, 2000; Stone *et al.*, 2000). Likewise, the cell death-inducing toxins fusicoccin from *Fusicoccum amygdali* or *Alternaria alternata* f.sp. *lycopersici* toxin from *Alternaria alternata* trigger expression of pathogenesis-related (*PR*) genes in tomato or *Arabidopsis*, respectively (Schaller and Oecking, 1999; Gechev *et al.*, 2004), whereas the host selective cell death-inducing toxin victorin from *Cochliobolus victoriae* was shown to elicit production of avenanthramide phytoalexins in oat (Tada *et al.*, 2005). In all cases studied so far, it remains unclear whether toxin-induced plant immune responses constitute an unavoidable consequence of toxin action or, alternatively, toxins have a second role as PAMPs that are perceived by plant PRRs.

Necrosis and ethylene-inducing protein 1 (Nep1)-like proteins (NLPs) are proteins for which this particular question has been addressed in more detail (Veit *et al.*, 2001; Fellbrich *et al.*, 2002; Qutob *et al.*, 2002; Mattinen *et al.*, 2004;

Pemberton *et al.*, 2005; Bae *et al.*, 2006). NLPs trigger a multifaceted plant immune response in various dicotyledonous plants, but not in monocotyledonous plants. NLPs are found in multiple bacterial, fungal and oomycete species, most of which favour a (hemi)biotrophic, necrotrophic or saprophytic lifestyle (Qutob *et al.*, 2006). NLPs are not present in higher eukaryotes including plants and were shown to be virulence factors in *Erwinia* spp. (Mattinen *et al.*, 2004; Pemberton *et al.*, 2005). Thus, NLPs appear to fulfil the criteria of a 'classical' PAMP. However, unlike PAMPs, NLPs are not 'on display' on the microbial surface but are produced strongly at later stages of infection. In the hemibiotroph oomycete *Phytophthora sojae*, NLP production was strongest during the transition from the biotrophic to the necrotrophic stage of infection (Qutob *et al.*, 2002). It is notable that a *Phytophthora*-derived NLP restored the virulence of NLP-deficient *Erwinia carotovora* mutants, suggesting that NLPs from both sources share the same molecular mode of action (our unpublished data). Other characteristics of NLPs further distinguish them from 'classical' PAMPs. In most PAMPS characterised, small 'antigenic' epitopes within the intact molecule were identified to be sufficient for the immunomodulatory activities of such molecules (Table 2.1). However, no such motif could be identified within various NLPs (Fellbrich *et al.*, 2002; Gijzen and Nürnberger, 2006). Moreover, NLPs cause cytolytic cell death in dicotyledonous plants that is genetically different from SA-dependent immunity-associated HR (Qutob *et al.*, 2006), while most PAMPs either do not induce HR at all or trigger SA-dependent HR (Kamoun, 2001). Notably, NLP-induced plant *PR* gene expression was SA-dependent (Fellbrich *et al.*, 2002). NLPs further disrupt plasma membrane vesicles prepared from dicot plants, but not those from monocot plants, indicating that an intact cell is not required for NLP-induced cell death (our unpublished data). Altogether these findings suggest that NLP-induced cell death is a disease symptom rather than a plant defence response. The cytolytic activity of NLPs and the broad spectrum of NLP-sensitive plants imply that these proteins are microbial toxins with immunostimulating potential.

Elucidation of the 3D structure of a *Pythium aphanidermatum* NLP (C. Oecking and H.U. Seitz, personal communication) and the computational modelling of additional NLP folds revealed a high degree of structural conservation between prokaryotic and eukaryotic NLPs. Moreover, NLP structures resemble proteinaceous cytolytic toxins, named actinoporins. Actinoporins bind to lipid docking sites in animal host target membranes, insert into membranes, form ion-conducting pores and subsequently lyse the target cells (Parker and Feil, 2005). Structure–activity relationship analyses performed on amino acid residues that are highly conserved among all NLPs suggested a close correlation between the ability of mutant proteins to cause cytolysis, restore virulence in NLP-deficient *Erwinia* and induce plant immune responses (our unpublished data). This is important since it indicates that a common fold of a cytolytic toxin mediates microbial attack and plant immunity. The mode of perception of NLPs on dicot plant membranes, however,

is currently unknown. It is conceivable that NLP-induced perturbation of host plasma membrane integrity may result in the release of a host-derived DAMP with immunomodulating activity. NLP-induced interference with host plasma membrane integrity may lead directly to the activation of immune responses in the affected cells in a similar way to that observed in plant cells treated with non-selective ionophores (Jabs *et al.*, 1997). Alternatively, NLPs may also act as PAMP-like triggers of plant immunity through binding to PRRs. In all cases, however, the plant could profit from recognition of a potentially fatal microbial virulence factor. It should be mentioned that several attempts to identify an NLP receptor (docking or binding site) by means of forward genetics have failed (M. Gijzen, personal communication; our unpublished data), suggesting that perception of these proteins is unlikely to be facilitated by non-redundant, non-essential, single gene-encoded receptors, such as the PRRs FLS2 or EFR (see below) (Gomez-Gomez and Boller, 2000; Zipfel *et al.*, 2006). Although PRR-mediated perception of NLPs cannot be excluded, it is also conceivable that NLPs may bind to plant targets that resemble toxin docking sites identified in animal membranes (Parker and Feil, 2005).

Toxin-induced immunity is not unique to plants since various bacteria-derived cytolytic toxins were shown to trigger both innate immune responses and cell death in mammalian cells (Huffman *et al.*, 2004; Srivastava *et al.*, 2005). For example, *Staphylococcus pneumoniae*-derived cytolytic pneumolysin triggered an immune response in mice that was mediated upon direct binding of the toxin to the PRR TLR4 (Srivastava *et al.*, 2005). In contrast, glycolipids found solely in insect and nematode plasma membranes can serve as docking sites for the cytolytic, pore-forming *Bacillus thuringiensis* (*Bt*) toxin and mediate membrane insertion and cell death (Griffitts *et al.*, 2005). Importantly, *Bt* toxin as well as another bacteria-derived pore-forming toxin, aerolysin, trigger immune responses in animal hosts (Huffman *et al.*, 2004). Altogether microbial toxins appear to stimulate animal innate immunity either upon recognition by PRRs or upon binding to cognate virulence targets on host cells.

Peptaibols, the products of non-ribosomal peptide synthetases, are linear peptide antibiotics produced by various fungal genera, including *Trichoderma*. These compounds are thought to contribute to the protection of fungi against bacterial infections (Engelberth *et al.*, 2000; Viterbo *et al.*, 2007). More than 300 different peptaibols have currently been identified and some were shown to possess plant immunity-stimulating potential (Viterbo *et al.*, 2007). The antibiotic functions of peptaibols have been assigned to their membrane insertion and pore-forming abilities (Engelberth *et al.*, 2000; Viterbo *et al.*, 2007). It is not known whether ion–pore formation is the trigger for immune activation in peptaibol-sensitive plants. Thus, these molecules may initiate the release of host-derived DAMPs or stimulate plant immune responses directly via their ionophore-like activity or upon recognition by yet unknown PRRs (similar to NLPs; see above).

2.5 PRRs in plant immunity

In vertebrate immune cells, PAMPs are predominately sensed by a class of receptors that resemble the *Drosophila* Toll protein and are therefore collectively called Toll-like receptors (TLRs) (Aderem and Ulevitch, 2000; Girardin *et al.*, 2002; Cook *et al.*, 2004; Akira *et al.*, 2006; Ferrandon *et al.*, 2007; Medzhitov, 2007). These PRRs are composed of extracytoplasmic leucine rich-repeat (LRR) domains, a transmembrane domain and a cytoplasmic TIR domain (*Drosophila* Toll and human interleukin-1 receptor) (Underhill and Ozinsky, 2002; Cook *et al.*, 2004).

Human cell types implicated in innate immunity (mucosal epithelia, phagocytes) express a total of 13 different TLRs, many of which recognise a multitude of microbe-derived PAMPs or host-derived DAMPs (Akira *et al.*, 2006; Ferrandon *et al.*, 2007; Medzhitov, 2007). For example, TLR4 recognises bacterial LPS, host-derived Hsp70 and hyaluronan, whereas TLR2 recognises approximately 15 microbial and host-encoded agonists (Akira *et al.*, 2006).

PAMP-binding sites have been detected in plasma membrane preparations from various plants but biochemical purification of these proteins proved notoriously difficult (Montesano *et al.*, 2003; Nürnberger *et al.*, 2004). The first successful purification of a PAMP-binding site was reported from soybean membranes, which recognised a specific molecular pattern, 1,6-β-linked, 1,3-β-branched heptaglucosides (HGs), from the cell walls of the phytopathogenic oomycete, *P. sojae* (Umemoto *et al.*, 1997; Mithöfer *et al.*, 1999). This 75-kDa HG-binding protein (HGP) conferred glucan binding to transgenic tobacco plants but surprisingly lacked any transmembrane or membrane attachment domain (Umemoto *et al.*, 1997). More recently, this protein was shown to harbour a hidden endoglucanase activity that released small oligomeric 1,3-β-D-oligoglucosides from complex glucans (Fliegmann *et al.*, 2004). Thus, it appears likely that plant glucanases may release oligoglucoside fragments from the oomycete cell wall that constitute suitable ligands for a yet unknown glucan receptor. The lack of functional domains for transmembrane signalling in HGP and the proposed existence of multimeric glucan recognition systems in Fabaceae (Mithöfer *et al.*, 1999) suggest that plant PAMP perception systems could be as complex as in animal cells (Akira *et al.*, 2006; Ferrandon *et al.*, 2007; Medzhitov, 2007).

The *Arabidopsis FLS2* (*fl*agellin *s*ensing 2) gene encodes a plasma membrane LRR-receptor kinase (LRR-RK) that recognises bacterial flagellin through its extracytoplasmic LRR domain (Gomez-Gomez and Boller, 2000; Chinchilla *et al.*, 2006). FLS2 is the only flagellin receptor in *Arabidopsis* because (i) the loss of FLS2 resulted in complete flagellin insensitivity (Gomez-Gomez and Boller, 2000), (ii) the presence of functional FLS2 correlated with flagellin sensitivity in all ecotypes tested (Bauer *et al.*, 2001) and (iii) the expression of *Arabidopsis* FLS2 in tomato conferred *Arabidopsis* flagellin signalling specificity to this plant (flagellin recognition specificities in tomato and *Arabidopsis* vary) (Robatzek *et al.*, 2007). Flagellin perception appears to be widespread

among the Solanaceae and Brassicaceae (Gomez-Gomez and Boller, 2002), but is lacking, for example, in the Umbelliferaceae (our unpublished data). Importantly, flagellin-induced immune responses were shown to restrict the growth of the adapted (virulent) *P. syringae* pv. *tomato* strain DC3000 (*Pst* DC3000), whereas *fls2* mutants were more susceptible to this pathogen (Zipfel *et al.*, 2004). Thus, bacterial pattern recognition through the PRR FLS2 contributes to basal plant immunity against adapted pathogens (restriction of growth) and likely to species immunity against non-adapted pathogens (halt of growth). This finding was significant because it provided the first evidence that PTI is indeed important for plant disease resistance. Inactivation of individual PRRs does not always result in measurable reduction of overall basal immunity (residual immunity in susceptible hosts) to adapted (virulent) pathogens. This is because the overall aggressiveness of virulent strains may simply override subtle effects brought about by PTI, an immunity that has been rendered insufficient by adapted pathogens. However, infection of plants lacking individual PRRs by partially 'disarmed' pathogenic strains, non-pathogenic strains (avirulent or TTSS-deficient strains) or non-adapted strains (for which a given plant species is not a host) may be suitable to demonstrate experimentally the role of these receptors in plant basal immunity.

The second important insight associated with the discovery of FLS2 became obvious only after the isolation of TLR5, the human flagellin receptor (Hayashi *et al.*, 2001). Besides the striking fact that flagellin perception exists in both lineages, a structural comparison between FLS2 and TLR5 revealed conservation of the modular structure in both receptors. Although primary sequences of plant FLS2 and human TLR5 differ considerably, both flagellin receptors carry extracytoplasmic LRR domains that are linked to a cytoplasmic portion. Whereas the cytoplasmic portion of FLS2 represents a functional serine/threonine protein kinase (PK) itself, the TIR domain of TLR5 forms a complex with the interleukin-1-receptor-associated kinase (IRAK) and the adaptor protein MyD88 (Hayashi *et al.*, 2001), and thereby links flagellin perception to cytoplasmic PK activity. Thus, the domain architecture of flagellin perception appears to be strongly conserved across kingdom borders. However, differences in the cytoplasmic domains of the animal and plant receptors suggest that these flagellin perception systems arose independently and are the result of convergent evolution rather than of divergent evolution. This view (Ausubel, 2005) is further corroborated (i) by the absence of LRR receptor-mediated immunity in unicellular eukaryotes (the supposed phylogenetic divergence point of animals and plants)h and (ii) by the fact that the antigenic epitopes within flagellin required for FLS2 activation (a conserved peptide motif within the N-terminal region of bacterial flagellin, flg22) and TLR5 (two helical structures within the central portion of flagellin) differ (Gomez-Gomez and Boller, 2002).

Plants possess approximately 235 LRR-RLKs (Shiu *et al.*, 2004) and a significant number of these are anticipated to serve as PRRs in PAMP perception

(Nürnberger and Kemmerling, 2006). This assumption is based on the findings that transcript levels of multiple LRR-RK-encoding genes increased upon pathogen infection or PAMP treatment. For example, bacterial infection of *Arabidopsis* plants resulted in enhanced expression of 49 LRR-RK-encoding genes (Kemmerling *et al.*, 2007), whereas flagellin treatment yielded increased transcript levels of 28 LRR-RK-encoding genes (Navarro *et al.*, 2004). Expression of flagellin-responsive *LRR-RLK* genes was also triggered by other PAMPs including bacterial LPS and fungal chitin (Zhang *et al.*, 2002; Thilmony *et al.*, 2006). The latter was particularly astonishing since it suggested that perception of different PAMPs trigger a generic plant response that potentially facilitates recognition of different microbial species.

Previously, Kunze *et al.* identified an N-terminal, acetylated 18-amino acid fragment (elf18) of *Escherichia coli* elongation factor Tu (EF-Tu) as a potent PAMP in *Arabidopsis* (Kunze *et al.*, 2004). Based upon identical response patterns observed in *Arabidopsis* seedlings treated with flg22 or elf18, it was assumed that the EF-Tu receptor might be structurally related to FLS2 (Zipfel *et al.*, 2006). By testing a collection of T-DNA insertion lines that are impaired in the expression of flg22-responsive *LRR-RK* genes, an elf18-insensitive mutant line was identified (Zipfel *et al.*, 2006). Insensitivity to elf18 was restored by ectopic expression of the *EFR* (*EF*-Tu *r*eceptor) gene in this mutant. Moreover, expression of the *EFR* gene in *Nb* conferred elf18 sensitivity that was lacking in *Nb* wild-type plants. EFR possesses an extracytoplasmic LRR domain that is linked to a cytoplasmic serine/threonine kinase domain and therefore closely resembles FLS2 (Gomez-Gomez and Boller, 2000; Zipfel *et al.*, 2006). Since *EFR* and *FLS2* fall into the same clade of *Arabidopsis LRR-RK* genes (LRR XII clade) (Shiu and Bleecker, 2001; Shiu *et al.*, 2004), it is predicted that other members of this clade encode 'orphan' PRRs that recognise as yet elusive microbial patterns. EFR-mediated sensing of bacteria did not contribute measurably to basal immunity of *Arabidopsis* against *Pst* DC3000 infections but limited *Agrobacterium tumefaciens*-mediated transformation of this plant (Zipfel *et al.*, 2006). Mutant lines defective in *efr* consistently showed approximately 50-fold higher expression levels of a *pBIN19-35S::GUS* reporter gene construct. This finding is notable from a biotechnological perspective because it suggests that suppression of PRR-mediated microbial pattern recognition could be a strategy to develop transformation protocols for crop plants that are recalcitrant to *Agrobacterium*-mediated transformation.

Regardless of the importance of LRR-RK type PRRs in plant immunity, other types of plant PRRs should also be considered (Nürnberger and Kemmerling, 2006; Bittel and Robatzek, 2007). For example, fungal chitin perception in rice is mediated through a plasma membrane LysM (lysin motif) receptor protein (LysM-P) that carries an extracytoplasmic LysM domain that is linked to a very small cytoplasmic domain (Kaku *et al.*, 2006). Biochemical evidence suggests that the LysM domain directly mediates binding of oligomeric chitooligosaccharide fragments (Ito *et al.*, 1997; Kaku *et al.*, 2006). It is currently unknown whether the very short cytoplasmic tail of this protein

contributes to the initiation of an intracellular signalling cascade. Alternatively, LysM-P may also form complexes with transmembrane proteins carrying cytoplasmic signalling domains. An *Arabidopsis* LysM-RK (CERK1) was recently implicated in chitin perception (Miya *et al.*, 2007). Mutants defective in the expression of the *CERK1* gene lacked any chitin-inducible immune responses, such as production of ROS (oxidative burst) or defence-related gene expression. It is unclear whether the LysM domain of CERK1 binds chitin physically and therefore it remains to be seen whether chitin perception and signalling are carried out by the same or distinct proteins. Altogether chitin recognition in plants appears to engage at least two types of LysM domain proteins: LysM-P and LysM-RK.

The LysM domain consists of approximately 40-amino acid residues and is originally known from a variety of bacterial enzymes involved in cell wall biosynthesis and degradation (Bateman and Bycroft, 2000). The finding that plant LysM domain-containing proteins bind fungal chitin (CERK1, plant chitinases) is interesting from an evolutionary point of view because the bacterial LysM domain is known as a general peptidoglycan-binding module that is present in a number of peptidoglycan-modifying enzymes such as *E. coli* murein transglycosylase D (Bateman and Bycroft, 2000). Peptidoglycans, also known as murein, are polymers consisting of sugars and amino acids that form a mesh-like layer in the cell walls of gram-negative and gram-positive bacteria. The sugar component consists of alternating residues of β-(1,4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues (a heteroglycan). Attached to the *N*-acetylmuramic acid is a peptide chain of three to five amino acids that can be cross-linked to the peptide chain of another strand forming a 3D mesh-like layer. Apparently, during evolution, plant LysM domain-containing proteins have acquired the ability to bind fungi-derived homoglycans consisting of β-(1,4)-linked *N*-acetylglucosamine (chitin) that are structurally related to the carbohydrate backbones of bacterial peptidoglycan. Moreover, the LysM motif is present in the *Lotus japonicus* lipochitooligosaccharide (Nod-factor) receptor kinases LjNFR1 and LjNFR2 that mediate the establishment of symbiosis between leguminous plants and rhizobacteria (Radutoiu *et al.*, 2003). This implies that plants utilise LysM domain receptors as a general module for chitin-based self/non-self-discrimination in both symbiotic and antagonistic plant–microbe interactions.

As peptidoglycans are potent triggers of immune responses in *Arabidopsis* and tobacco (Felix and Boller, 2003; Gust *et al.*, 2007), it will be important to test whether plant LysM domain-containing proteins may (in addition to their ability to sense fungal chitin fragments) also recognise and respond to this microbial pattern. At least six peptidoglycan perception systems in humans [TLR2, NOD1 (nucleotide-binding oligomerisation domain 1), NOD2 and *Drosophila* immune cells (PGRP (peptidoglycan recognition protein)-SA, PGRP-LC, PGRP-SC1B)] are known to recognise different fragments of bacterial peptidoglycans (Akira *et al.*, 2006; Ferrandon *et al.*, 2007). However, none of these proteins is a LysM-P or LysM-RK, suggesting that LysM-mediated

carbohydrate recognition in eukaryotic innate immunity may be found primarily in plants.

Recognition of a fungal xylanase and subsequent activation of cultivar non-specific immunity in tomato was ascribed to a plasma membrane-anchored extracellular LRR-protein (LRR-P) that lacks a cytoplasmic signalling domain (Ron and Avni, 2004). As plant genomes harbour multiple LRR-P-encoding sequences (the *Arabidopsis* genome contains 56 LRR-P-encoding sequences), it is conceivable that these proteins constitute another class of PRRs that are mechanistically similar to animal LRR-P-type PAMP receptors (Nürnberger and Kemmerling, 2006; Bittel and Robatzek, 2007). Whether ligand perception and intracellular signal transduction through plant LRR-Ps (similar to LysM-P) requires additional components is currently not known.

2.6 Intracellular signal transduction in PTI

PAMP-mediated activation of PRRs conveys ligand-encoded information across the plasma membrane, thereby initiating a host-signalling cascade that culminates in the activation of pathogen non-specific immune responses. Previous pharmacological evidence suggested that PK activity is required to trigger very rapid PAMP responses in plants such as influxes of H^+ and Ca^{2+} across the plasma membrane (Felix *et al.*, 1991, 1999; Nürnberger *et al.*, 1994; Felix and Boller, 2003). Remarkably, the *fls2-17* allele of *FLS2* carries a point mutation in the PK domain that is responsible for complete flagellin insensitivity and loss of autophosphorylation activity of FLS2 in vitro (Gómez-Gómez *et al.*, 2001). Surprisingly, this mutation also negatively affected binding of flg22 to the extracytoplasmic LRR domain of FLS2, suggesting that overall stability or conformation of the receptor was affected. A mutation in a putative phosphorylation site of FLS2 also caused flagellin insensitivity, which again emphasises the importance of PK activity for the activation of flagellin-inducible plant responses (Robatzek *et al.*, 2006). Moreover, overexpression of KAPP, a kinase-associated protein phosphatase that is believed to regulate the function of several transmembrane RKs, resulted in flagellin insensitivity and reduced flagellin binding to FLS2 (Gómez-Gómez *et al.*, 2001) (Fig. 2.2). Altogether available evidence suggests that phosphorylation of FLS2 (by its own intrinsic PK activity or by another yet unknown PK) is a crucial element of flagellin sensing/signalling.

Very recently, BAK1 (BRI1-associated receptor kinase 1), an LRR-RK that controls plant growth by heterodimerisation with the plant brassinosteroid (BR) hormone receptor, BRI1 (an LRR-RK itself) (Wang *et al.*, 2001; Li *et al.*, 2002), was implicated in FLS2 and EFR function (Chinchilla *et al.*, 2007). *Bak1* mutants were (partially) insensitive to both flg22 and elf18, albeit to different degrees. Flg22-dependent rapid heterodimerisation of FLS2 and BAK1 was further demonstrated by co-immunoprecipitation experiments (Chinchilla *et al.*, 2007) (Fig. 2.2), suggesting a similar mode of action of BAK1 in FLS2

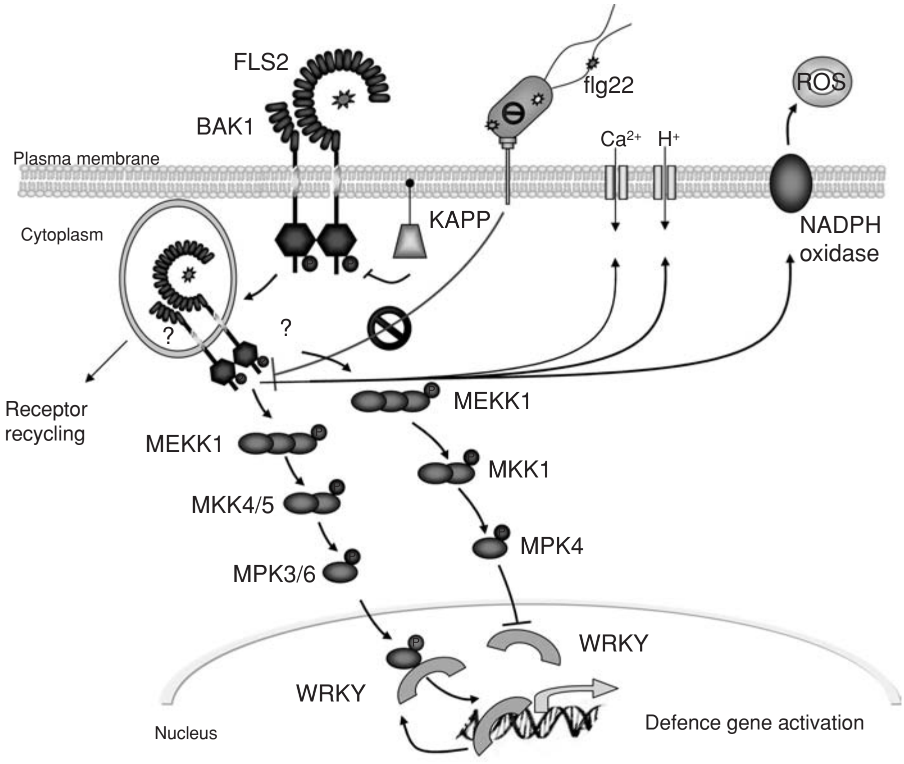

Figure 2.2 Flagellin perception and signal transduction in *Arabidopsis*. The bacteria-associated molecular pattern flagellin (flg22) is recognised by the pattern recognition receptor FLS2. Ligand-induced heterodimerisation with the LRR-RK BAK1 is required for activation of plant cellular responses, including proton influx of protons (extracytoplasmic alkalinisation), increase in cytoplasmic calcium levels, production of ROS (oxidative burst), posttranslational activation of MAP kinase cascades and WRKY transcription factor-mediated gene expression. The MEKK1/MKK4/5/MPK3/6 cascade is a positive regulator of flg22-inducible genes, whereas the MEKK1/MKK1/MPK4 cascade regulates flg22-dependent gene expression in a negative manner. Ligand-induced internalisation of FLS2 into endosomal compartments might be required for receptor clearance and intracellular signalling.

and BRI1 activation. In addition to its role as positive regulator of PTI and plant growth, BAK1 appears to have additional functions. *Bak1* mutants were also shown to have altered disease-resistance phenotypes to biotrophic and necrotrophic pathogens that are likely the consequence of infection-induced de-regulated cell death control (Kemmerling *et al.*, 2007). Thus, in addition to its role as a positive regulator of PTI, BAK1 may further act as a negative regulator of plant cell death. Both plant immunity-associated functions of BAK1 are independent of BR activity because various other mutants impaired

in BR sensitivity or biosynthesis were not impaired in flagellin sensitivity or infection-induced runaway cell death (Chinchilla *et al.*, 2007; Kemmerling *et al.*, 2007). Thus, BAK1 has BR-independent, immunity-associated functions in addition to its well-established BR-dependent role in plant development (Morillo and Tax, 2006). BAK1 represents a second example of a plant LRR-RK with dual functions in plant development and immunity. Previously, the receptor kinase ERECTA was implicated in both flower development and plant pathogen resistance (Godiard *et al.*, 2003; Llorente *et al.*, 2005). Dual roles for receptor proteins in development and immunity are also known from animal systems. A precedence is provided by the *Drosophila* receptor TOLL that controls embryonic patterning and immunity against fungal infections in adult insects (Lemaitre *et al.*, 1996).

Attenuation and termination of PRR function in animal immunity is achieved mainly by the activities of negative regulators. In *Arabidopsis*, KAPP-mediated inactivation of FLS2 has been proposed to be such a mechanism (Gómez-Gómez *et al.*, 2001). More recently, proteasome-dependent, ligand-induced endocytosis of FLS2 was demonstrated and proposed to be an additional way to shut down PRR activity in plant immunity (Robatzek *et al.*, 2006). In addition, internalisation of FLS2 into endosome-like compartments may contribute to flg22 signalling (Fig. 2.2).

Signalling pathways implicated in PTI employ altered cytoplasmic Ca^{2+} levels, ROS, nitric oxide (NO) and several mitogen-activated protein kinase (MAPK) cascades (Zhang and Klessig, 2001; Jonak *et al.*, 2002; Nürnberger *et al.*, 2004) (Fig. 2.2). Strikingly, most of these components are important for PAMP-induced activation of innate immune responses in animal cells (Barton and Medzhitov, 2003), suggesting further conservation in the molecular basis of innate immunity in eukaryotes. NO production has been observed both in PAMP-treated plants and during ETI in resistant host plants (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Clarke *et al.*, 2000). Although there is no conclusive evidence for a plant enzyme that is homologous to human NO synthase (hNOS), pharmacological hNOS inhibitors blocked infection and elicitor-stimulated NO production, cell death and defence gene activation in plants (Delledonne *et al.*, 1998; Durner *et al.*, 1998). Zeidler *et al.* reported that AtNOS1, a plant-specific NOS previously associated with hormone signalling in plants (Guo *et al.*, 2003), mediated LPS-induced NO production and *PR* gene expression in *Arabidopsis* (Zeidler *et al.*, 2004). Importantly, inactivation of the *AtNOS1* gene not only abrogated LPS-induced NO production in these plants but also dramatically enhanced susceptibility of the mutant to *Pst* DC3000 infection. Recently, the purported role of AtNOS1 as a bona fide plant NO synthase was questioned as the 3D-fold of the protein suggested similarities to mitochondrial GTPase involved in mitochondrial biogenesis (Zemojtel *et al.*, 2006). Thus, it appears likely that reduced levels of NO in *AtNOS1* mutants and the observed immunity-associated phenotypes are likely due more to mitochondrial dysfunction affecting NO production (mitochondria are a major source of NO) than to a lack of NO synthase activity (Zemojtel *et al.*,

2006). NO-mediated protein nitrosylation has been found in PAMP-treated plant cells (Lindermayr *et al.*, 2005, 2006). However, a regulatory role of nitrosylated proteins in the activation of PTI is lacking.

MAPKs are central points of crosstalk in plant-signalling cascades including those that protect against microbial invasion (Zhang and Klessig, 2001; Gomez-Gomez and Boller, 2002; Jonak *et al.*, 2002; Nakagami *et al.*, 2005). Various fungi- or bacteria-derived PAMPs and phytopathogenic microbes activate MAPK enzyme activities in a transient manner. In particular, *Arabidopsis* AtMPK3 and AtMPK6 are responsive to PAMP treatment or infection (Zhang and Klessig, 2001; Jonak *et al.*, 2002). Notably, silencing of *MPK6* resulted in compromised disease resistance in *Arabidopsis* (Menke *et al.*, 2004). In PAMP-treated parsley cells, PcMPK3 and PcMPK6 translocate to the nucleus (Ligterink *et al.*, 1997; Lee *et al.*, 2004) and contribute to WRKY transcription factor-dependent *PR* gene expression (Eulgem *et al.*, 1999; Kroj *et al.*, 2003). Using an *Arabidopsis* protoplast transient expression system, Asai *et al.* (2002) identified a flg22-induced MAPK cascade (made of the MAPK kinase kinase MEKK1, the MAPK kinases MKK 4/5 and MAPK 3/6) and WRKY transcription factors that act downstream of FLS2 and proposed a role of this cascade in bacterial and fungal resistance (Asai *et al.*, 2002). PAMP-triggered MAPK pathways appear to exert both positive and negative regulation of PTI-associated responses in plants. Recently, flg22 was shown to activate the MEKK1/MKK1/MPK4 pathway that suppresses various pathogen defence pathways including callose deposition and *PR* gene expression (Ichimura *et al.*, 2006; Suarez-Rodriguez *et al.*, 2007). Thus, fine-tuning of PTI-associated responses is achieved through at least two MAPK pathways that exert positive and negative regulatory activities. Surprisingly, flg22-induced activation of the MPK4 pathway did not require MEKK1 kinase activity, suggesting that the protein may function as a scaffold or structural protein (Suarez-Rodriguez *et al.*, 2007).

The only known substrates that are directly phosphorylated by AtMPK6 are two isoforms of 1-aminocyclopropane-1-carboxylic acid synthase (ACS), the rate-limiting enzyme of ethylene biosynthesis (Liu and Zhang, 2004). Phosphorylation of ACS2 and ACS6 by MPK6 led to the accumulation of ACS protein, elevated levels of cellular ACS activity, ethylene production and ethylene-induced plant phenotypes. Causal links between MAPK activation, expression of *PR* genes and initiation of programmed cell death were suggested by a set of loss- and gain-of-function experiments performed in tobacco or *Arabidopsis*, respectively (Zhang and Klessig, 2001; Jonak *et al.*, 2002; Ren *et al.*, 2002; Nürnberger *et al.*, 2004).

Surprisingly, little is known about the role of plant hormones in the activation of PTI-associated immune responses. Classical hormones implicated in the establishment of ETI, such as salicylic acid, jasmonic acid or ethylene, appear not to be required for some flg22-induced defence responses and basal immunity to *Pst* DC3000 (Zipfel *et al.*, 2004). Thus, although flg22 triggers ethylene biosynthesis in *Arabidopsis* (Felix *et al.*, 1999), this appears not to be

needed for the activation of PTI in PAMP-treated cells. However, it might contribute to the activation of defences in plant cells remote from the site of PAMP application. Flagellin treatment further induces a plant micro-RNA that negatively regulates transcript levels of auxin receptors TIR1, AFB2 and AFB3 (Navarro *et al.*, 2006). Moreover, repression of auxin signalling restricted growth of *Pst* DC3000, suggesting that the growth-promoting hormone, auxin, is a disease susceptibility factor and that miRNA-mediated suppression of auxin signalling is an element of PTI. Suppression of auxin-mediated growth as a consequence of activated immunity might reflect the functionality of an innate trade-off between plant immunity and growth programs.

Expression of genes encoding several components implicated in basal immunity of host plants or species resistance is enhanced by various PAMPs (Bittel and Robatzek, 2007). Gene products, such as PEN1, PEN2, PEN3, EDS1 and PAD4, are thus likely to contribute to PAMP-triggered immune responses in infected plants, although mutants in these genes have so far not been reported to display obvious defects in responses to individual PAMPs.

2.7 Suppression of PTI by bacterial effectors

Suppression of PTI is a major strategy of virulent pathogens to facilitate infections in susceptible host plants (see also Chapter 3). The first report of this phenomenon showed that TTSS-deficient mutants of *Pst* DC3000 (incapable of effector delivery into host cells) triggered a number of plant defences, such as callose apposition and *PR* gene expression, that were suppressed by virulent *Pst* DC3000 (Hauck *et al.*, 2003). Activation of these responses by TTSS-deficient bacteria was proposed to be mediated through PAMP perception/PRR activities. Later, such responses were indeed reported to be triggered by various PAMPs (He *et al.*, 2007). Among the first bacterial effectors shown to suppress PTI-associated responses were AvrPto and AvrPtoB (He *et al.*, 2006). In a screen conducted to identify *Pst* effectors that suppress PAMP-inducible MAPK activation and flg22-induced early gene expression in *Arabidopsis* protoplasts, both effectors (but not several others) were found to block PAMP-induced signal transduction cascades upstream of MAPK activities (He *et al.*, 2006). In these experiments, it remained unclear whether these effectors inhibited a regulator of PTI pathways or interfered directly with components of PAMP-triggered signalling pathways. The 3D-structure-based identification of AvrPto as a Ser/Thr PK inhibitor (Xing *et al.*, 2007) favoured the idea that soluble PKs and/or cytoplasmic PK domains of transmembrane LRR-RKs could serve as direct targets for bacterial effector activities *in planta*. Indeed, AvrPto was recently shown to bind to BAK1 in vivo, thereby inhibiting flg22-induced heterodimerisation of FLS2 and BAK1 (L. Shan, P. He and J. Sheen, personal communication). As BAK1 is implicated in the function of additional PRRs such as EFR (Chinchilla *et al.*, 2007), interference

with BAK1 may be a powerful strategy pursued by virulent pathogens to suppress PTI and to infect susceptible host plants (Fig. 2.2).

Another molecular mode of interference with PTI was proposed for *P. syringae* effectors AvrRpt2 and AvrRpm1 that inhibit PAMP-induced signalling and compromise host basal immunity through manipulation of a regulator of PTI, RIN4 (Kim *et al.*, 2005). *Arabidopsis* RIN4 is targeted by the cysteine protease AvrRpt2 for proteolytic degradation (Mackey *et al.*, 2003; Coaker *et al.*, 2005) and by AvrRpm1 for phosphorylation-dependent inactivation (Mackey *et al.*, 2002). Strikingly, resistant *Arabidopsis* ecotypes that harbour the resistance (R) proteins RPS2 and RPM1 develop AvrRpt2/AvrRpm1 ETI that is based upon R protein-mediated sensing ('guarding') of a manipulation of PTI by these effectors (Mackey *et al.*, 2002, 2003). Arguably, this is one of the nicest examples of a mechanistic link between both forms of plant immunity (PTI/ETI). The evolution of effectors that target PTI provides further evidence for the physiological relevance of PAMP-triggered immune responses.

Bacterial entry into plant tissues often occurs through stomata and is a critical step of the infection process. In an elegant study, Melotto *et al.* (2006) demonstrated that abscisic acid-dependent stomatal closure observed upon bacterial infection is due to PRR-mediated PAMP perception. Infection of *Arabidopsis* with TTSS-deficient *Pst* DC3000 resulted in FLS2-dependent stomatal closure. Importantly, initial closure of stomata was also observed upon infection by *Pst* DC3000 but was reversed at later times of infection, suggesting that virulent pathogens have evolved means to reopen closed stomata. The authors further reported that stomatal reopening correlated with the TTSS-independent activity of the bacterial toxin coronatine (Melotto *et al.*, 2006). The evolution of several types of effectors (secreted toxins and TTSS effectors delivered into the plant cell) again demonstrates the importance of PTI suppression in establishment of infection and microbial proliferation on host plants.

2.8 Concluding remarks

There is now strong evidence that plants and animals deploy innate immune (PTI) systems that share a similar logic (Nürnberger *et al.*, 2004; Ausubel, 2005; Zipfel and Felix, 2005; Chisholm *et al.*, 2006; Jones and Dangl, 2006). Self/non-self-discrimination is based on the detection of invariant, microbe-specific patterns by host-encoded PRRs. As a result, species-specific antimicrobial defences are triggered that can halt microbial growth. Moreover, both plants and animals appear to possess sensor systems that facilitate recognition of host-derived signals representing damaged or non-intact self. Common principles in the organisation of innate immunity in both lineages are further exemplified by the nature of microbial patterns that are recognised, similar PRR types and related signalling cascades. However, significant differences in the molecular organisation of immunity in plants and animals remain. Plant

cells respond to microbial infection in a cell-autonomous manner, whereas in animals, specialised cell types protect host tissues against microbial invasion (Jones and Dangl, 2006; Medzhitov, 2007). Moreover, adaptive immunity that evolved in jawed vertebrates and most likely arose as a result of insufficient innate immunity (Medzhitov, 2007) is missing in plants (Ausubel, 2005). In plants, insufficiency of PTI mediated, for example, by suppressive activities of microbial effectors has likely driven the evolution of ETI (Alfano and Collmer, 2004; Chisholm *et al.*, 2006). Although there is currently no experimental evidence that supports the latter assumption, there is evidence that PTI is an important element of basal immunity against adapted pathogens and of species immunity against non-adapted pathogens (Nürnberger and Lipka, 2005; Bittel and Robatzek, 2007). Moreover, resistance protein-mediated ETI was recently shown to operate in one case through a de-repression and potentiation of PAMP-inducible gene expression, thus suggesting a functional interdependence between the two types of plant immunity (Shen *et al.*, 2007).

Research during the last decade has arguably provided an unprecedented gain of knowledge in the field of plant immunity in general, and PTI research in particular. Major information gaps remain to be filled. The identification of new PRRs and their corresponding ligands, the elucidation of host receptors for DAMP signals (endogenous elicitors) and a detailed understanding of toxin-induced immunity are to name just a few.

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Chapter 3

PSEUDOMONAS SYRINGAE TYPE III-SECRETED PROTEINS AND THEIR ACTIVITIES AND EFFECTS ON PLANT INNATE IMMUNITY

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Abstract: Bacterial pathogens of both plants and animals use type III protein secretion systems (T3SSs) to inject bacterial proteins called type III effectors into host cells to alter host physiology and favor the pathogen. The bacterial plant pathogen *Pseudomonas syringae* is dependent on the Hrp T3SS to cause disease on plants. It has become apparent over the past several years that a primary target for *P. syringae* type III effectors is the plant innate immune system. The innate immune system of plants and animals use specific pattern recognition receptors (PRRs) to recognize conserved molecules of microorganisms called pathogen-associated molecular patterns (PAMPs) and induce a basal defense now known as PAMP-triggered immunity (PTI). Several *P. syringae* type III effectors have been shown to suppress PTI. A second tier of the plant innate immune system uses resistance (R) proteins to detect pathogen effectors, including bacterial type III effectors, and induce an immune response now known as effector-triggered immunity (ETI). Several *P. syringae* type III effectors can also suppress ETI. The existence of R protein-recognized type III effectors and type III effectors that suppress ETI and PTI have produced a molecular arms war of sorts in which the bacterial pathogen has evolved an assortment of offensive type III effectors that suppress plant immunity and plants a myriad of defensive PRRs and R proteins that detect the presence of pathogens. This chapter covers what is known about proteins that are secreted by the *P. syringae* T3SS. In addition to type III effectors, type III-secreted helper proteins assist in type III effector delivery. Since a primary target of type III effectors is the plant innate immune system, an introduction to it is also included. Recent progress suggests that *P. syringae* type III effectors can be used as tools

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to identify new components of plant immunity. Increasing our understanding of the activities and targets of type III effectors will likely reveal how plants defend themselves against bacterial invasion.

Keywords: bacterial effectors; type III secretion; pattern recognition receptors; PAMP-triggered immunity; host defence suppression

3.1 Introduction

Pseudomonas syringae is a bacterial plant pathogen that can live in the extracellular space in-between plant cells called the apoplast (Alfano and Collmer, 1996; Preston, 2000). It is a member of the gamma subgroup of Proteobacteria that contains several animal pathogenic bacteria (e.g. *Salmonella*, *Shigella* and *Yersinia* spp.) as well as several important plant pathogenic bacteria, including *Erwinia*, *Pantoea*, *Xanthomonas* and *Xylella* (Gupta, 2000). *P. syringae* has been separated into greater than 50 pathovars based on the plants that a strain can infect. These plants include virtually every agricultural crop. In host plants, *P. syringae* can grow to high cell densities and causes chlorotic and necrotic lesions. The strain *P. syringae* pv. *tomato* DC3000 has been a popular strain to study because it is pathogenic on tomato, an important crop plant, and the genetically amenable model plant *Arabidopsis* (Whalen *et al.*, 1991; Kunkel, 1996). The genome of DC3000 has been sequenced (Buell *et al.*, 2003), and more recently the sequence of the genomes of two other strains, *P. syringae* pv. *phaseolicola* 1448A and *P. syringae* pv. *syringae* B728a, is now known (Joardar *et al.*, 2005).

The ability of *P. syringae* to be pathogenic is dependent on the Hrp type III protein secretion system (T3SS). This system is truly remarkable in that it has the ability to inject bacterial proteins called type III effectors into plant cells. While most of the activities and plant targets of these effectors are not well understood, the emerging picture is that these effectors suppress plant innate immunity (Espinosa and Alfano, 2004; Abramovitch *et al.*, 2006). The Hrp T3SS was originally discovered through random mutagenesis of *P. syringae* and the collection of mutants isolated were called *hrp* mutants because they lost the ability to elicit a hypersensitive response (HR), a programmed cell death (PCD) response associated with resistant plants, as well as the ability to be pathogenic on susceptible plants (Lindgren *et al.*, 1986). These mutants turned out to have mutations in genes encoding proteins needed for the T3SS apparatus. Thus, they were unable to secrete any type III effectors into plant cells. This showed that *hrp* genes were required for nonhost plants to induce defenses in response to *P. syringae* and for disease on host plants. It would be about another 7 years before researchers discovered that *hrp* genes encoded a protein secretion system (Van Gijsegem *et al.*, 1993).

Another interesting class of genes discovered during the 1980s, which later turned out to be related to *hrp* genes, is the bacterial avirulence (*avr*) genes.

These genes were typically discovered by expressing DNA libraries derived from a strain that is avirulent on a particular plant host in a virulent strain that causes disease on that host (Staskawicz *et al.*, 1984). This approach resulted in a collection of *avr* genes, which when expressed in virulent strains blocked the ability of virulent strains to be pathogenic. It was not until the mid-1990s that we discovered that Avr proteins are actually type III effector proteins that are injected by the T3SS where they are recognized by resistance (R) proteins inside the plant cell (Alfano and Collmer, 1996).

The Hrp T3SS of *P. syringae* secretes two different classes of proteins – the already mentioned type III effectors that are injected into plant cells by the T3SS, and extracellular accessory proteins that we will refer to here as helper proteins. Included in the helper protein class are all those proteins that function outside the bacterial cell to facilitate the injection of type III effectors. This includes the HrpA1 pilus protein, which is the main component of the Hrp pilus (Roine *et al.*, 1997; Jin and He, 2001; Li *et al.*, 2002); the HrpK1 protein, which is a putative translocator (defined below) (Petnicki-Ocwieja *et al.*, 2005) and the harpins HrpZ1 and HrpW1, which were two of the earliest proteins discovered to be secreted by the *P. syringae* T3SS (He *et al.*, 1993; Charkowski *et al.*, 1998). The roles of the harpins have been ambiguous for some time, but recent data suggest that they may act on the plant cell wall and/or the plasma membrane as translocators. Translocators are a class of helper protein that forms pores in the plant plasma membrane and, through an unknown mechanism, facilitates the delivery of type III effectors into plant cells (Buttner and Bonas, 2002).

The separation of the type III-secreted proteins into two classes is based on their known or predicted function and their site of action. However, it remains possible that a protein that does not act inside plant cells is still an effector that is targeting a plant protein outside the plant cell. The secretion signal for both classes of proteins is carried on their N-termini. While there is no conserved secretion signal, there are biochemical characteristics that are shared by all *P. syringae* type III-secreted proteins (Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002). Thus, it is worth noting that the secretion signals for both classes appear to be the same. In fact, the HrpW1 harpin, a protein predicted to target the plant cell wall, was identified as an injected protein (Guttman *et al.*, 2002). This may indicate that all type III-secreted proteins can be injected into plant cells even if they function outside, or that helper proteins also function as effectors inside the plant cell. Alternatively, it may indicate that the translocation assays currently being used cannot distinguish the helper proteins from the injected effectors.

In this chapter, we discuss activities of *P. syringae* type III-secreted proteins by mainly focusing on type III effectors and their targets inside plant cells. Current knowledge of *P. syringae* helper proteins is also presented. If the reader is interested in type III effectors from other bacterial plant pathogens, several recent reviews include these topics (Mudgett, 2005; Abramovitch *et al.*, 2006; Chisholm *et al.*, 2006; Grant *et al.*, 2006; Angot *et al.*, 2007; Speth *et al.*,

2007). In order to cover adequately the roles of *P. syringae* type III effectors, we include a brief introduction to the plant innate immune system, which is a primary target of many type III effectors.

3.2 *P. syringae* type III-secreted helper proteins

The Hrp T3SS apparatus is encoded by the *hrp/hrc* genes within the Hrp pathogenicity island (Alfano *et al.*, 2000). The *hrc* genes encode T3SS apparatus proteins that are conserved in all bacteria containing T3SSs (Bogdanove *et al.*, 1996). For many of the genes with the *hrp* designation, it is still unknown what role they perform in type III secretion. However, it seems likely that they are either components of the T3SS apparatus or members of the helper protein class. Recently, several *hrp* genes were shown to encode proteins that are secreted via the T3SS (Fu *et al.*, 2006; Ramos *et al.*, 2007). These include HrpJ, HrpB, HrpD, HrpF and HrpP. While not being similar enough to broadly conserved T3SS proteins to qualify for the Hrc designation, all these proteins (with the exception of HrpF) are similar to proteins in animal pathogen T3SSs (Hueck, 1998; Pallen *et al.*, 2005). We are beginning to know more about the HrpJ protein. *P. syringae* pv. *tomato* DC3000 mutants lacking HrpJ are unable to secrete the harpins HrpZ1 and HrpW1 (Fu *et al.*, 2006; A.D. Karpisek and J.R. Alfano, unpublished work), suggesting that HrpJ acts as a control protein for harpin secretion.

In pioneering research on T3SSs, HrpA1 was shown to be the major component of a Hrp pilus, a tube that extends from the base of the Hrp T3SS apparatus (Roine *et al.*, 1997; Jin and He, 2001; Li *et al.*, 2002). Animal pathogenic T3SSs generally have a shorter appendage referred to as the T3SS needle (Cornelis, 2006). In plant pathogen T3SSs, the needle is replaced by the Hrp pilus, which is significantly longer than animal pathogens' needles. The longer length of the Hrp pilus in plant pathogen T3SSs may be required for the apparatus to cross the plant cell wall and reach the plant plasma membrane. It was first shown with the *P. syringae* Hrp pilus that these extracellular appendages (needles and Hrp pili) act as conduits for type III-secreted proteins (He and Jin, 2003).

The translocation of type III effectors into eukaryotic cells requires proteins that can form pores in the eukaryotic plasma membrane. These proteins have been studied extensively in animal pathogens and generally consist of three proteins that interact with each other to form a translocon (Buttner and Bonas, 2002; Cornelis, 2006). Two of these proteins (e.g. *Yersinia* YopB and YopD) have hydrophobic domains that can form pores in artificial membranes. The third protein is a hydrophilic protein that is found at the tip of the animal pathogen's T3SS apparatus (e.g. *Yersinia* LcrV) (Mueller *et al.*, 2005; Picking *et al.*, 2005).

In plant pathogen T3SSs, the process of translocation is much less well understood. A putative translocator, HrpF, was identified in *Xanthomonas*

campestris (Buttner *et al.*, 2002). HrpF was shown to be capable of forming pores in membranes and was required for the translocation of type III effectors into plant cells, but not for secretion of them in culture. The *P. syringae* HrpK1 protein has weak similarity to HrpF and it also is required for the translocation of effectors into plant cells, but not secretion of type III effectors in culture (Petnicki-Ocwieja *et al.*, 2005). Even though the experiments testing whether HrpK1 could form pores in membranes have not been performed, based on the secretion and translocation phenotypes of *P. syringae hrpK1* mutants and the weak similarity with HrpF, HrpK1 is a putative translocator. Does the translocation process in plant pathogens occur substantially different than in animal pathogens? The presence of the plant cell wall suggests that translocation may occur significantly differently in plant–pathogen interactions. Also, the genes encoding translocators in animal pathogens are generally adjacent to each other and therefore relatively easy to identify. No other obvious candidate translocator genes are adjacent to the *hrpK1* gene in *P. syringae*. Does HrpK1 act alone or are there other proteins that function with HrpK1 in a translocon analogous to animal pathogen T3SSs? Presently the answer to this question is unknown. However, recently the HrpZ1 and HrpW1 harpins have become candidate translocators.

The harpins of phytopathogenic bacteria, including the *P. syringae* HrpZ1 harpin, were the first proteins found to be secreted by Hrp T3SSs (Wei *et al.*, 1992; He *et al.*, 1993; Arlat *et al.*, 1994). Originally, they were thought to be proteins responsible for host range because when purified and infiltrated into plant tissue they elicited an HR. However, it required relatively high amounts of purified harpin for HR-eliciting activity and therefore the HR elicited by harpins probably does not represent the HR elicited by avirulent bacteria. However, it is possible that harpins can be recognized by plants, perhaps as a pathogen-associated molecular pattern (PAMP) (discussed further below), but the levels secreted by bacteria are too low to elicit an HR. Indeed, the *P. syringae* HrpZ1 harpin was found to bind to a nonproteinaceous binding site in tobacco plasma membranes consistent with it being recognized by a receptor (Lee *et al.*, 2001a).

But what is the virulence role for harpins? There are many properties of harpins that suggest that they have a role in translocation. *P. syringae* HrpZ1 was shown to bind plant cell walls (Hoyos *et al.*, 1996) and later it was found to be able to form pores in artificial membranes (Lee *et al.*, 2001b) consistent with it being a translocator. *P. syringae* HrpW1 has a pectate lyase domain that can bind to pectate (Charkowski *et al.*, 1998). Recently, two other *P. syringae* HrpW1-like harpins, HopAK1 and HopP1, have been described (Kvitko *et al.*, 2007). Interestingly, a *P. syringae* quadruple harpin mutant lacking HrpZ1, HrpW1, HopAK1 and HopP1 was reduced in translocation (Kvitko *et al.*, 2007) and when *hrpK1* was also mutagenized, making a *P. syringae hrpK1*/quadruple harpin mutant, translocation was further reduced (Kvitko *et al.*, 2007). Importantly, all these proteins (excluding HopP1) were capable of partially restoring translocation, suggesting that HrpZ1, HrpW1

and HopAK1 are functionally related to the putative translocator HrpK1 (Kvitko *et al.*, 2007). While there is still much unknown about translocation in *P. syringae*, recent data suggest that translocation requires proteins that modify the plant cell wall together with proteins that form pores in the plant plasma membrane.

3.3 The plant innate immune system

Innate immunity is the first line of defense in higher plants and animals against microorganisms that could potentially harm them. Both plants and animals recognize the presence of microorganisms by detecting conserved molecules called pathogen-associated molecular patterns (PAMPs), which are also known as microbe-associated molecular patterns (MAMPs), because they are generally not unique to pathogens (Janeway and Medzhitov, 2002; Ausubel, 2005; Mackey and McFall, 2006). Detection of PAMPs occurs through receptors termed pattern recognition receptors (PRRs) and this triggers signal transduction pathways that result in PAMP-induced immunity (PTI) in plants to distinguish it from another layer of the immune system called effector-triggered immunity (ETI) (see Fig. 3.1) (Chisholm *et al.*, 2006; Jones and Dangl, 2006) (see also Chapter 2). With bacterial pathogens, ETI occurs when a specific type III effector is recognized. The effectors recognized are generally specific to pathogens and are not present in nonpathogens. Historically, recognized type III effectors were referred to as bacterial avirulence (Avr) proteins (Leach and White, 1996). In ETI, type III effectors are recognized by specific R proteins. There is much overlap between ETI and PTI in terms of their outputs and the signal transduction pathways involved. In general, ETI is a more acute response that often involves the HR, a PCD response associated with resistance, whereas PTI usually does not. Both PTI and ETI trigger common signal transduction cascades including calcium influx and activation of mitogen-activated protein kinase (MAPK), pathways followed by the expression of defense genes, and this results in outputs that limit the growth of microorganisms (Nurnberger *et al.*, 2004; Zipfel and Felix, 2005). Plants also employ hormone signals to adapt to biotic and abiotic stresses, and type III effectors seem to be able to modulate these responses (de Torres-Zabala *et al.*, 2007; Loake and Grant, 2007; Robert-Seilaniantz *et al.*, 2007). In addition, plants send out a systemic warning signal to the rest of the plant when local infections occur, called systemic acquired resistance to protect plant tissues that were not directly assaulted by pathogens (Durrant and Dong, 2004) (see also Chapter 11).

3.3.1 PAMP-triggered immunity

Plants and animals have evolved a highly successful strategy to defend against microbes by evolving a surveillance system that recognizes conserved

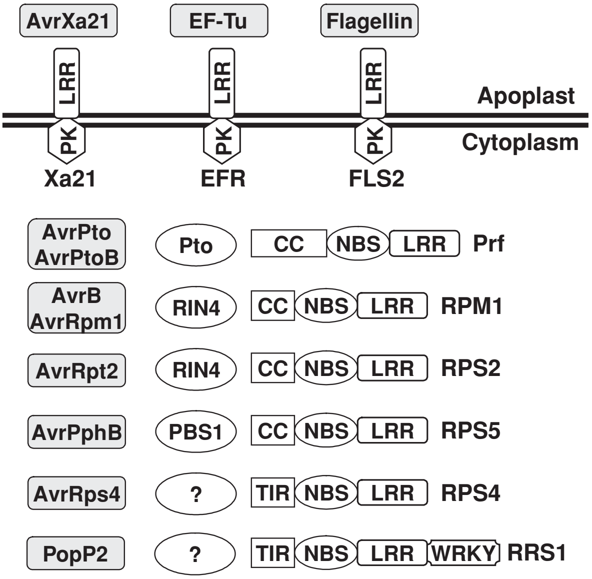

Figure 3.1 Representative plant pattern recognition receptors (PRRs) and resistance (R) proteins that recognize bacterial molecules or activities. The Xa21 receptor-like kinase recognizes an unidentified bacterial signal, the receptor-like kinase EF-Tu receptor (EFR) recognizes the elongation factor EF-Tu and the FLS2 receptor kinase recognizes flagellin. These PRRs have an extracellular leucine-rich repeat (LRR) domain and an intracellular Ser/Thr protein kinase (PK) domain. Representative R proteins are shown that possess a nucleotide-binding site (NBS) domain, an LRR domain and either a coiled-coil (CC) domain or a Toll and IL-1 receptor (TIR) domain. These R proteins recognize the indicated bacterial type III effectors directly or indirectly. Also, indicated are plant proteins, where known, that are involved in the recognition of specific type III effectors. See text for more details.

molecules (i.e. PAMPs) of microbes (that are not easily lost or altered) to trigger innate immune responses. PAMPs recognized by plants can be proteins, polysaccharides or lipids, and include fungal chitin, ergosterol and β-glucan; heptaglucosides and Pep-13 from oomycetes; and bacterial flagellin, cold stress protein, elongation factor Tu (EF-Tu), peptidoglycan and lipopolysaccharide (Nurnberger *et al.*, 2004; Zipfel and Felix, 2005).

Flagellin is one of the most extensively studied PAMPs and is considered to be an important PAMP in both plants and animals (Nurnberger *et al.*, 2004). Moreover, failure to recognize flagellin renders the host, whether plant or animal, more susceptible to pathogens (Zipfel *et al.*, 2004; Andersen-Nissen *et al.*, 2007), highlighting the importance of flagellin perception in PTI. Flagellin is perceived by the receptor kinase FLAGELLIN SENSING 2 (FLS2) in *Arabidopsis* (Chinchilla *et al.*, 2006). FLS2 is a transmembrane receptor kinase containing an extracellular leucine-rich repeat (LRR) domain and an intracellular kinase domain and is a member of the subfamily LRR-XII of receptor-like

kinases (Shiu *et al.*, 2004) (see Fig. 3.1). This is the best-characterized PRR in plants and is present in many plant species (Felix *et al.*, 1999). For example, an FLS2 homolog was recently identified in *Nicotiana benthamiana* (Hann and Rathjen, 2007). FLS2 is critical for PTI since plants that have mutations or knockdowns that stop or reduce *FLS2* expression display reduced PAMP responses and increased susceptibility to pathogens (Zipfel *et al.*, 2004; Hann and Rathjen, 2007). Flagellin perception appears to be evolutionarily conserved in plants and animals. However, in animals PRRs that function on the outside of the cell to sense PAMPs are Toll-like receptors (TLRs that have an intracellular Toll domain), including the TLR5 PRR that perceives flagellin, whereas plants use transmembrane receptor kinases to recognize PAMPs such as flagellin. Thus, the different types of PRRs that recognize the same PAMP appear to have been derived through convergent evolution (Ausubel, 2005).

Another PAMP whose PRR receptor kinase has been identified is the bacterial EF-Tu (Kunze *et al.*, 2004). This protein is the most abundant protein in bacteria and is critical for protein translation. The receptor that recognizes EF-Tu in *Arabidopsis* is the receptor-like kinase EF-Tu receptor (EFR), similar in structure to FLS2 (Kunze *et al.*, 2004; Zipfel *et al.*, 2006). EF-Tu from *Escherichia coli* induces PAMP responses, including medium alkalization within minutes of addition to an *Arabidopsis* cell culture as well as other hallmarks of PTI such as the activation of MAPKs and restriction of bacterial growth (Kunze *et al.*, 2004; Zipfel *et al.*, 2006). Synthetic EF-Tu peptides originating from different sources (e.g. other bacteria) are also capable of inducing PAMP responses (Kunze *et al.*, 2004). These responses are significantly more efficient than those induced by plant organellar EF-Tu, suggesting that plants have evolved to distinguish their own molecules from nonself, which would be required if an organism focused on a broadly conserved protein as a PAMP. Moreover, the EF-Tu from *P. syringae* elicits a weaker PTI response than the EF-Tu from *Agrobacterium tumefaciens*, suggesting that *P. syringae* may have altered its EF-Tu protein to avoid detection by EFR-dependent PTI (Zipfel *et al.*, 2006). The similarity of the EFR and FLS2 receptors may indicate that they share a common signaling pathway. In fact, co-treatment of flagellin and EF-Tu peptides does not show additive or synergistic effects in PAMP responses and they appear to activate a similar set of genes (Zipfel *et al.*, 2006). Expression of the *EFR* and *FLS2* genes are induced as a result of PTI, suggesting positive feedback regulation (Zipfel *et al.*, 2004, 2006), perhaps to induce a stronger immune response against subsequent pathogen attacks.

3.3.2 Effector-triggered immunity

ETI represents a second layer of innate immunity and is triggered by effector proteins of plant pathogens. Bacterial type III effectors generally trigger ETI after they have been injected by the T3SS and recognized inside the plant

cell by R proteins. One exception to this is likely to be mediated by the R protein Xa21, which is a transmembrane receptor kinase similar to FLS2 that recognizes an unidentified compound secreted by the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* outside the plant cell (Song *et al.*, 1995; da Silva *et al.*, 2004). Historically, *R* genes and type III effector genes formed the basis of the gene-for-gene hypothesis to explain race-cultivar resistance in plants (Keen, 1990). The vast majority of R proteins contain a central nucleotide-binding site (NBS) domain and a C-terminal LRR domain. NBS-LRR R proteins can be further divided based on the domain present in their N-termini. This N-terminal domain is either a coiled-coil (CC) domain or a Toll and IL-1 receptor (TIR) domain (see Fig. 3.1). Most NBS-LRR R proteins that perceive bacterial type III effectors are CC-NBS-LRR R proteins. However, the *Arabidopsis* R protein RPS4, which recognizes the *P. syringae* type III effector AvrRps4, is a TIR-NBS-LRR R protein (Gassmann *et al.*, 1999). An unusual member of the TIR-NBS-LRR type R protein group is the *Arabidopsis* R protein RRS1 that possesses a C-terminal tryptophan-arginine-lysine-tyrosine (WRKY) domain (Deslandes *et al.*, 2003). Proteins with WRKY domains are predicted to act as transcription factors (Eulgem *et al.*, 2000), implying that this particular R protein modifies transcription. RRS1 recognizes the *Ralstonia solanacearum* type III effector PopP2 and after recognition both the R protein and the type III effector localize to the plant nucleus, a site consistent with the WRKY domain's predicted function. The existence of RRS1 seemed an anomaly until the recent discovery that other R proteins have the capacity to localize to the plant nucleus and interact with plant transcription factors (Burch-Smith *et al.*, 2007; Shen *et al.*, 2007). An emerging idea from these interesting recent papers is that R proteins may have additional roles in innate immunity beyond effector recognition.

The tomato R protein Pto was the first R protein isolated that recognizes the presence of a pathogen effector. It is a Ser/Thr kinase (i.e. not an NBS-LRR R protein) required for the recognition of the *P. syringae* effectors AvrPto1 and AvrPtoB (Martin *et al.*, 1993; Tang *et al.*, 1996; Kim *et al.*, 2002). Quite soon after the discovery of Pto, a typical CC-NBS-LRR type R protein was found to be required for Pto-mediated immunity (Salmeron *et al.*, 1996). Because Prf was an NBS-LRR type R protein, it seemed plausible that Prf was the key recognition component in Pto/Prf-mediated immunity. This dichotomy led to the development of the 'guard hypothesis' to explain why Pto would require Prf to trigger innate immunity (Van der Biezen and Jones, 1998; Dangl and Jones, 2001; Innes, 2004; Jones and Dangl, 2006). A central point of the guard hypothesis is that NBS-LRR R proteins 'guard' a virulence target, a host target that the pathogen would benefit from by modifying. However, when the pathogen's effector modifies the virulence target, the NBS-LRR R protein that is guarding or monitoring it detects this modification and thereby induces ETI. If the guard hypothesis holds, several predictions can be made about the host target: Modifying the target should benefit the pathogen in a susceptible plant, alteration of the guarded target in a resistant plant should induce ETI

and it seems likely that the target would be conserved broadly in susceptible plants. Conceptually, Prf guarding a Pto virulence target makes sense with the known immune functions of other NBS-LRR R proteins. However, in the case of Pto and Prf, several points argue against the guard hypothesis. For example, tomato plants lacking Pto are not known to be more susceptible to *P. syringae* and Pto is not a broadly conserved protein. However, the recent discovery that AvrPto1 acts as an inhibitor of the Pto kinase is consistent with Prf, sensing the AvrPto1–Pto interaction à la guard and guardee (Xing *et al.*, 2007). While it remains unknown whether Pto and Prf are functioning as guardee and guard, respectively, the guard hypothesis has unveiled many testable questions that can be addressed through experimentation in the future.

The FLS2 PRR recognizes flagellin by direct physical interaction (Chinchilla *et al.*, 2006). There are only a few examples where NBS-LRR R proteins perceive pathogen effectors directly (Jia *et al.*, 2000; Deslandes *et al.*, 2003; Dodds *et al.*, 2006). Other NBS-LRR proteins recognize effectors indirectly and this provides support for the guard hypothesis (Mackey *et al.*, 2002, 2003; Axtell and Staskawicz, 2003; Shao *et al.*, 2003; Rooney *et al.*, 2005). One example comes from analysis of the *Arabidopsis* NBS-LRR R proteins RPS2 and RPM1, the *P. syringae* type III effectors AvrRpt2, AvrB and AvrRpm1, and *Arabidopsis* RIN4 protein. RPS2 recognizes AvrRpt2, while RPM1 recognizes both AvrB and AvrRpm1, inducing ETI. RIN4 interacts with both RPM1 and RPS2 R proteins as well all three type III effectors (Mackey *et al.*, 2002, 2003; Axtell and Staskawicz, 2003). AvrRpt2 is a cysteine protease that degrades RIN4 and this event somehow induces RPS2-dependent ETI (Axtell *et al.*, 2003; Axtell and Staskawicz, 2003; Mackey *et al.*, 2003). Likewise, both RPM1 and RPS2 induce the phosphorylation of RIN4 and, while it has not been reported, this may be the signal that induces RPM1-dependent ETI (Mackey *et al.*, 2002). Thus, it appears that RIN4 is a target of these three type III effectors and their corresponding R proteins perceive different modifications of RIN4.

3.3.3 Outputs of PTI and ETI

After PTI or ETI occurs, signal transduction pathways are employed that result in transcriptional changes in the plant. Both PTI and ETI utilize MAPK pathways to induce gene expression (Asai *et al.*, 2002; Jin *et al.*, 2002). Microarray studies indicate that there is much overlap between PTI and ETI in terms of gene expression profiling (Tao *et al.*, 2003; Navarro *et al.*, 2004; Zipfel *et al.*, 2004). Salicyclic acid (SA) is considered as a major signaling compound for ETI, while PTI has both SA-dependent and -independent responses. A recent report indicates that SA levels increase in *Arabidopsis* after treatment with a PAMP and that *Arabidopsis* mutants defective in SA biosynthesis are affected in PTI based on expression profiling (Tsuda *et al.*, in press). These results suggest that SA plays a signaling role in PTI.

There is also overlap in the defense outputs from ETI and PTI. These outputs include callose deposition, phytoalexin synthesis, reactive oxygen species and nitric oxide production, changes in cytoplasmic Ca^{2+}, production of pathogen-related gene expression and the HR. As noted above, the HR is more closely associated with ETI than PTI. The failure of PTI to induce the HR may rather reflect more of a threshold phenomenon than different signaling mechanisms. For many innate immunity outputs, it is not known whether they are effective against pathogens individually because they are induced together. For example, callose deposition is a thickening of the cell wall localized at the sites of pathogen ingress (Brown *et al.*, 1993). It can also be induced by purified PAMPs such as flagellin (Gomez-Gomez *et al.*, 1999). Many type III effectors can suppress this response. However, it is not known how callose affects bacteria. Apparently, it can protect plants from bacterial pathogens since an *Arabidopsis* mutant defective in callose biosynthesis allowed significantly more growth of T3SS defective bacteria than do wild-type plants (Kim *et al.*, 2005). In earlier studies, the same *Arabidopsis* mutant was more resistant to a fungal pathogen than was wild-type *Arabidopsis* (Nishimura *et al.*, 2003), suggesting that fungal pathogens have evolved to take advantage of this type of cell wall apposition.

3.4 Enzymatic activities and plant targets of *P. syringae* type III effectors

There were clues in the early 1990s that the T3SSs of plant pathogens were capable of suppressing plant innate immunity. For example, Jakobek *et al.* (1993) showed that a virulent *P. syringae* pathovar, *P. syringae* pv. *phaseolicola*, suppressed induction of defense-related mRNA and phytoalexins in bean that were separately induced by an avirulent (i.e. recognized through ETI) *P. syringae* pathovar and nonpathogenic *E. coli*. These early studies suggested that the T3SS of bacterial pathogens is involved in suppression of innate immunity. More recent studies suggest that individual type III effectors can alter innate immunity (Jackson *et al.*, 1999; Tsiamis *et al.*, 2000). Several *P. syringae* effectors have been identified as suppressors of the HR and other responses associated with innate immunity (Abramovitch *et al.*, 2003; Axtell and Staskawicz, 2003; Bretz *et al.*, 2003; Espinosa *et al.*, 2003; Mackey *et al.*, 2003; Jamir *et al.*, 2004; Lopez-Solanilla *et al.*, 2004; Kim *et al.*, 2005; Janjusevic *et al.*, 2006; Nomura *et al.*, 2006). A subset of these effectors was shown to suppress other hallmarks of innate immunity (Bretz *et al.*, 2003; Chen *et al.*, 2004; Jamir *et al.*, 2004). Moreover, several *P. syringae* type III effectors suppressed PCD in yeast (Abramovitch *et al.*, 2003; Jamir *et al.*, 2004). While the mechanism of suppression is unknown, suppression of PCD pathways in yeast suggests that these effectors may act on conserved pathways in eukaryotes.

Type III effectors are able to suppress defenses triggered by PAMPs (i.e. PTI). For example, Hauck *et al.* (2003) found that transgenically expressed AvrPto1 in *Arabidopsis* suppressed the expression of a set of genes predicted to encode proteins that are secreted cell wall and defense proteins that are normally expressed in an SA-independent manner. Other effectors suppress SA-dependent responses (DebRoy *et al.*, 2004). Moreover, when *P. syringae* T3SS defective mutants were sprayed onto transgenic *Arabidopsis* plants expressing specific type III effectors, these mutants grew to significantly higher levels than did control strains, suggesting that plant defenses induced by these mutants were suppressed by these type III effectors (Hauck *et al.*, 2003; Chen *et al.*, 2004). While the majority of *P. syringae* effectors studied appear capable of suppressing plant innate immunity, the enzymatic activities and targets within the plant cell for most of these remain unknown. Table 3.1

Table 3.1 *P. syringae* type III effectors and their enzymatic activity and/or plant targets[a]

Type III effector	Enzymatic activity	Plant target	Reference
AvrB and AvrRpm1	Possible kinases	RIN4	Mackey *et al.* (2002)
AvrPphB	Papain-like cysteine protease	PBS1 kinase	Shao *et al.* (2002) and Shao *et al.* (2003)
AvrPto1	Kinase inhibitor	Pto kinase	Xing *et al.* (2007)
AvrPtoB	E3 ubiquitin ligase	Fen kinase	Janjusevic *et al.* (2006) and Rosebrock *et al.* (2007)
AvrRpt2	Staphopain-like cysteine protease	RIN4 and others	Axtell *et al.* (2003), Axtell and Staskawicz (2003) and Mackey *et al.* (2003)
HopAI1	Phosphothreonine lyase	MAPKs	Li *et al.* (2007) and Zhang *et al.* (2007)
HopAO1	Protein tyrosine phosphatase	MAPK?	Bretz *et al.* (2003) and Espinosa *et al.* (2003)
HopI1	DNA J domain-containing protein	Hsp70?	Jelenska *et al.* (2007)
HopM1	26S proteasome	AtMIN7 and others	Nomura *et al.* (2006)
HopN1	Papain-like cysteine protease	Unknown	Lopez-Solanilla *et al.* (2004)
HopU1	ADP-ribosyltransferase	GRP7 and other RNA-binding proteins	Fu *et al.* (2007)

[a] See text for more details.

lists the *P. syringae* type III effectors for which the enzymatic activity and/or plant targets are known, and Fig. 3.2 summarizes the activities and targets of various *P. syringae* effectors inside the plant cell.

3.4.1 Type III effectors associated with phosphorylation or dephosphorylation

The *P. syringae tomato* DC3000 effector HopAO1 (formerly named HopPtoD2) contains a C-terminal protein tyrosine phosphatase (PTP) domain and possesses PTP activity. DC3000 mutants lacking HopAO1 are reduced in their ability to grow in plants and HopAO1 has been shown to suppress innate immune responses, including the HR, reactive oxygen species production and *PR1* expression (Bretz *et al.*, 2003; Espinosa *et al.*, 2003). The PTP active site was required for HopAO1's suppressor activity and *hopAO1* mutants could not be complemented with a construct encoding a catalytically inactive derivative of HopAO1 (Espinosa *et al.*, 2003). Because HopAO1 was able to suppress an HR-like response induced by a constitutively active mitogen-activated protein kinase kinase, it was hypothesized that HopAO1 may target MAPKs involved in innate immunity signal transduction (Espinosa *et al.*, 2003). However, a recent report showed that HopAO1 was unable to dephosphorylate MPK3 and MPK6 MAPKs (Underwood *et al.*, 2007), suggesting that HopAO1 functions downstream of MAPK pathways.

One *P. syringae* pv. *tomato* DC3000 type III effector that is active on MAPKs is HopAI1. This effector belongs to the same family as the *Shigella flexneri* type III effector OspF (Li *et al.*, 2007). Both HopAI1 and OspF are threonine-specific MAPK phosphothreonine lyases that remove phosphates from threonine residues within MAPKs. However, the mechanism that these enzymes use to accomplish this are fundamentally different from dephosphorylation by phosphatases (Li *et al.*, 2007) and it should be noted that dephosphorylation by lyases is not reversible in contrast to the reversible phosphate removal by phosphatases. HopAI1 has been shown to bind plant MAPKs (MPK3 and MPK6) and suppress PAMP-induced MAPK activation and other PTI responses (Li *et al.*, 2007; Zhang *et al.*, 2007).

In contrast to the dephosphorylation activities of HopAO1 and HopAI1, AvrRpm1 and AvrB cause the direct or indirect hyperphosphorylation of RIN4. RIN4 is a negative regulator of innate immunity and was identified based on its interaction with AvrB and the R protein RPM1 (Mackey *et al.*, 2002). Subsequently, RIN4 was also found to interact with the RPS2 R protein (Mackey *et al.*, 2003). AvrRpm1 and AvrB share little sequence similarity but are both recognized by RPM1 (Grant *et al.*, 1995). These effectors are both myristoylated in the host and localized to the plant plasma membrane (Nimchuk *et al.*, 2000). Interestingly, both AvrRpm1 and AvrB can suppress PTI (Kim *et al.*, 2005). Because these effectors cause the phosphorylation of RIN4, it is tempting to speculate that they are kinases. The structure of AvrB alone and bound with a RIN4 fragment has been determined (Lee *et al.*,

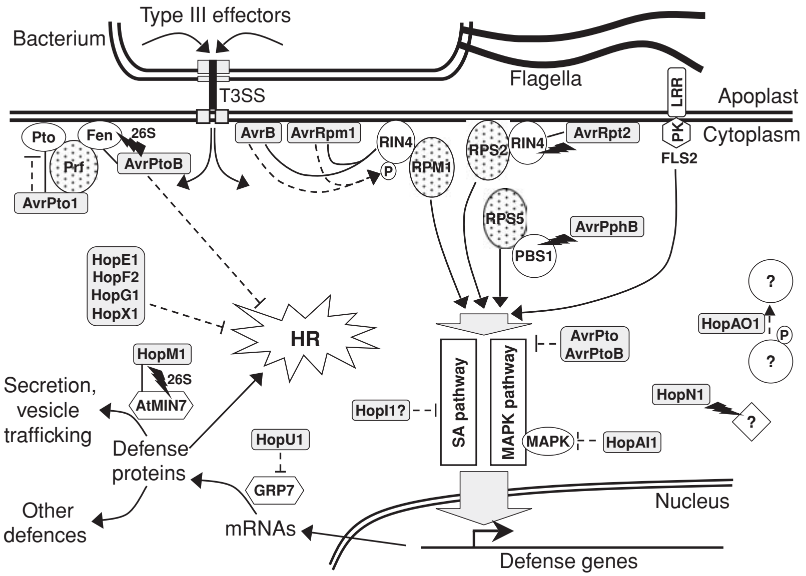

Figure 3.2 The activities and plant targets of *Pseudomonas syringae* type III effectors and their effects on plant innate immunity. Type III effectors (depicted as shaded rectangles) are translocated into plant cells via the type III protein secretion system (T3SS). Specific type III effectors can induce effector-triggered immunity (ETI). For example, the nucleotide-binding site leucine-rich repeat (NBS-LRR) type resistance proteins Prf, RPM, RPS2 and RPS5 (depicted as stippled ovals) detect the presence of AvrPto1 and AvrPtoB, AvrRpm1 and AvrB, AvrRpt2, and AvrPphB, respectively. Bacteria can also be perceived by the plant through recognition of pathogen-associated molecular patterns (PAMPs), such as recognition of flagellin by the FLS2 receptor kinase, and induce PAMP-triggered immunity (PTI). After ETI or PTI is induced, signal transduction pathways are employed often involving mitogen-activated protein kinase (MAPK) and salicyclic acid (SA)-dependent or -independent pathways, resulting in transcriptional changes in the plant. The production of immunity-related mRNAs results in outputs of innate immunity. Some of these outputs likely require vesicle trafficking to transport defense compounds outside the plant cell at sites of pathogen ingress. ETI generally results in elicitation of the HR. AvrPto1 was recently shown to inhibit the Pto kinase, while AvrPtoB possesses E3 ligase activity and initiates the ubiquination of the Fen kinase, marking it for degradation via the 26S proteasome (depicted with lightening rods with 26S). AvrRpm1 and AvrB cause either directly or indirectly the hyperphosphorylation of RIN4. The AvrRpt2 and AvrPphB effectors using cysteine protease activities directly degrade (depicted with lightening rods) RIN4 and PBS1, respectively. AvrPto1 and AvrPtoB were reported to suppress PTI through the inhibition of MAPK signaling, while HopAI type III effector can dephosphorylate MAPKs with its phosphothreonine lyase activity. HopM1 interacts with and leads to the Ub of AtMIN7, causing its degradation via the 26S proteasome. HopU1 ADP-ribosylates GRP7 and other RNA-binding proteins in vitro, which suggests that it suppresses innate immunity by affecting RNA metabolism. HopI1 localizes to the chloroplast and can affect SA production in certain genetic backgrounds. HopAO1 is an active protein tyrosine phosphatase, while HopN1 is an AvrPphB-like cysteine protease. However, their plant targets are currently unknown. Other type III effectors are known to suppress either PTI or ETI but their activities and targets are unknown. Direct interactions between type III effectors and plant proteins are depicted with solid lines; host pathways are indicated by solid arrows; dashed arrows are used to indicate type III effector activities. See text for more details.

2004; Desveaux *et al.*, 2007). AvrB does possess a nucleotide-binding pocket, and its structure, when bound to the RIN4 fragment, resembles Ser/Thr kinases (Desveaux *et al.*, 2007). However, no kinase activity has been detected with purified AvrB, leaving this attractive hypothesis unconfirmed. Another recent report suggests that AvrB interacts directly with the RAR1 protein, an HSP90 co-chaperone required for R protein function, suggesting that AvrB may target multiple proteins (Shang *et al.*, 2006).

3.4.2 An ADP-ribosyltransferase type III effector

Mono-ADP-ribosyltransferases (ADP-RTs) are well-characterized toxins in animal pathogens and are used in mammals as regulatory proteins (Morrison *et al.*, 2006; Yates *et al.*, 2006). However, until recently they were not known to be active in plant pathogens or plants. Bioinformatic investigations of the *P. syringae* pv. *tomato* DC3000 genome revealed that there were three type III effector genes encoding proteins resembling ADP-RTs (Collmer *et al.*, 2002). One of these effector proteins, HopU1 (formerly named HopPtoS2), was recently shown to be an active ADP-RT, and *Arabidopsis* substrates have been identified in vitro (Fu *et al.*, 2007). HopU1 can suppress both PTI and ETI in a manner that is dependent on its ADP-RT catalytic active site. Proteomic screening of its enzymatic targets has identified several RNA-binding proteins belonging to a family of RNA-binding proteins that possess an RNA recognition motif. An *Arabidopsis* T-DNA mutant lacking one of these substrates, GRP7 (glycine-rich RNA-binding protein 7) – which contains an RNA recognition motif at the N-terminus and a glycine-rich domain at the C-terminus, exhibited enhanced disease symptoms, increased susceptibility to the pathogen and decreased PTI responses (Fu *et al.*, 2007). GRP7 was known to be associated with circadian rhythms where it regulates its own mRNA level over the course of the day (Carpenter *et al.*, 1994; Heintzen *et al.*, 1997; Staiger and Apel, 1999; Staiger *et al.*, 2003). One could consider day/night change an abiotic stress, albeit a weak one. Thus, the involvement of GRP7 in other stress responses such as pathogen stress would not be inconsistent with what is known about this protein. In fact, the initial identification of *GRP7* was based on its induction upon stress (van Nocker and Vierstra, 1993; Carpenter *et al.*, 1994), and GRP7 was also found to provide protection to cold stress when heterologously expressed in bacteria (Kim *et al.*, 2007). It remains to be determined whether GRP7 interacts specifically with immunity-related RNA or has a more general role in RNA metabolism.

3.4.3 Type III effectors that affect ubiquination and/or the 26S proteasome

Bacterial type III effectors have been shown to eliminate host proteins by degrading them directly using their protease activity (discussed below) or indirectly by hijacking the host's protein degradation system, the 26S

proteasome. Proteins are marked for 26S proteasome-mediated degradation by the attachment of multiple ubiquitin (Ub) proteins linked covalently via their lysine 48 residues (Pickart and Fushman, 2004). The ubiquination process requires the activities of several enzymes: a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2) and a Ub-protein ligase enzyme (E3). The E3 enzyme controls the specificity of this reaction by recruiting the target protein to be ubiquinated and marking it for degradation. An emerging theme among bacterial pathogens of plants and animals is co-opting this stage of the Ub pathway to degrade host proteins to favor pathogenesis (Angot *et al.*, 2007).

The *P. syringae* pv. *tomato* DC3000 type III effector AvrPtoB (also known as HopAB2) contains an E3 ligase domain in its C-terminal half (Janjusevic *et al.*, 2006; Rosebrock *et al.*, 2007). Significantly, C-terminal truncations of AvrPtoB lacking the E3 ligase domain cause bacteria, expressing it to be recognized by certain tomato varieties, leading to a resistance phenomenon called Rsb (for resistance suppressed by AvrPtoB C terminus) (Abramovitch *et al.*, 2003; Rosebrock *et al.*, 2007). Thus, the C-terminal E3 ligase domain of AvrPtoB is capable of suppressing an immune response triggered by the N-terminal portion of AvrPtoB. This leads to the prediction that an R protein recognizes the N-terminal half of AvrPtoB and that the E3 ligase domain of AvrPtoB can suppress its recognition. Interestingly, the Fen kinase and Prf proteins were shown to be required for the Rsb response (Rosebrock *et al.*, 2007). Fen belongs to the same family as the Pto kinase which is required for the recognition of both AvrPto1 and full-length AvrPtoB, and both Fen- and Pto-mediated immunity responses are Prf-dependent (Pedley and Martin, 2003; Lin *et al.*, 2006). Indeed, the E3 ligase domain of AvrPtoB was shown to interact directly with Fen kinase and cause Fen to be ubiquintated, promoting its degradation via the 26S proteasome (Rosebrock *et al.*, 2007). One attractive hypothesis is that AvrPtoB acquired an E3 ligase domain to suppress a Fen/Prf-dependent immune response and at a later point the plant countered this by evolving another kinase, Pto, to recognize the full-length AvrPtoB (Rosebrock *et al.*, 2007). Of course, this is assuming that Prf is not guarding Fen and Pto and that both of these proteins are functioning as R proteins. If one tries to align these recent findings in a manner consistent with the guard hypothesis, Fen and Pto may both be virulence targets that are guarded by Prf. A slightly more complicated working of the guard hypothesis could have Fen and Pto as decoy targets that are guarded by Prf even though they themselves are not actual virulence targets. Thus, the 'real' virulence target would presumably look similar to these Ser/Thr kinases.

AvrPtoB can also suppress other immune responses that are independent of Pto and Fen and are induced by other recognized effectors, suggesting that AvrPtoB has multiple activities and plant targets (Abramovitch *et al.*, 2003; Jamir *et al.*, 2004). In addition, AvrPtoB disrupts the flagellin PAMP–FLS2 pathway and suppresses its immunity outputs in *Arabidopsis* and *N. benthamiana* (Akira *et al.*, 2006; Hann and Rathjen, 2007). Therefore, AvrPtoB appears to be able to suppress both PTI and ETI.

Another *P. syringae* type III effector HopM1 (formerly named HopPtoM) also appears to co-opt the 26S proteasome and degrade host proteins for the pathogen's benefit. While most *P. syringae* type III effector mutants are reduced only subtly in virulence, a DC3000 mutant lacking a portion of the conserved effector locus (CEL) displayed a substantial reduction in disease symptoms and the ability to multiply *in planta* (Alfano *et al.*, 2000). This phenotype was attributed to the type III effectors *avrE* and *hopM1* (Badel *et al.*, 2003; DebRoy *et al.*, 2004). Yeast two-hybrid analyses using N-terminal portions of HopM1 as bait found several *Arabidopsis* interacting proteins, named AtMIN proteins (*Arabidopsis thaliana* HopM1 interactors). Eight of the AtMIN proteins were not present in yeast when co-expressed with full-length HopM1, suggesting that HopM1 caused their degradation in yeast and a similar phenotype was observed when they were expressed with full-length HopM1 *in planta* (Nomura *et al.*, 2006). Subsequently, it was shown that the degraded host proteins were polyubiquinated and that degradation of these proteins was dependent on the 26S proteasome (Nomura *et al.*, 2006). *Arabidopsis* T-DNA mutants corresponding to each of the AtMIN proteins restricted the growth of the ΔCEL mutant except the AtMIN7 mutant which allowed the ΔCEL mutant to grow to higher levels than it did in wild-type plants, suggesting that AtMIN7 is targeted by HopM1. AtMIN7 is an ADP-ribosylation factor guanine exchange factor (ARF-GEF), which are key components of vesicle trafficking in eukaryotes, suggesting that this ARF-GEF may be involved in the secretion of defense-related molecules to the apoplast. HopM1 does not have any molecular signature that would obviously link it to the Ub pathway or the 26S proteasome. Therefore, it may function as an adaptor protein that recruits proteins to the 26S proteasome machinery (Nomura *et al.*, 2006; Speth *et al.*, 2007).

3.4.4 Cysteine protease type III effectors

Cysteine protease activity has emerged as a relatively common enzymatic activity possessed by many type III effectors from both animal and plant pathogens, including *P. syringae* (Shao *et al.*, 2002). AvrRpt2 is a staphopain-like cysteine protease that is active on several *Arabidopsis* proteins, including RIN4 (Axtell and Staskawicz, 2003; Mackey *et al.*, 2003; Chisholm *et al.*, 2005). The degradation of RIN4 is somehow detected by the RPS2 R protein-inducing ETI (Axtell *et al.*, 2003; Axtell and Staskawicz, 2003; Mackey *et al.*, 2003). Similarly, AvrPphB is a type III effector that cleaves *Arabidopsis* PBS1 with its papain-like cysteine protease activity (Shao *et al.*, 2003), and can be detected by the NBS-LRR R protein RPS5 (Ade *et al.*, 2007). Molecular analysis of the AvrPphB–PBS1–RPS5 interaction provides additional experimental support for the guard hypothesis.

While we know at least one target for both AvrRpt2 and AvrPphB, we do not know how these targets favor virulence in plants lacking their R protein guards. RIN4 is a negative regulator of flagellin-induced PTI and plants

lacking it are more resistant to pathogens, not less resistant. If RIN4 is a virulence target, the expectation would be that plants facking it would be more susceptible to the pathogen (Mackey *et al*., 2002; Kim *et al*., 2005). However, its central role in ETI and the fact that multiple type III effectors target it suggest that its elimination could be beneficial to pathogens. AvrRtp2 must also have other virulence targets because bacterial strains expressing AvrRpt2 grow to higher levels on *Arabidopsis* plants lacking RIN4 and RPS2 (Belkhadir *et al*., 2004; Lim and Kunkel, 2004). Similarly, an *Arabidopsis pbs1* mutant is not obviously more susceptible to *P. syringae* than is wild-type *Arabidopsis*, suggesting that it is not AvrPphB's sole virulence target if it is one at all (Warren *et al*., 1999). Another papain-like cysteine protease is the HopN1 (formerly named HopPtoN) type III effector identified in *P. syringae* pv. *tomato* DC3000. HopN1 has been demonstrated to be an active cysteine protease that can suppress plant PCD (Lopez-Solanilla *et al*., 2004). Its plant targets remain unknown.

3.4.5 Other notable *P. syringae* type III effectors

HopI1 (formerly named HopPmaI) was shown to localize in the chloroplast in a noncanonical manner and disrupt thylakoid structures, leading to enhanced virulence. HopI1 contains a J domain that is present in proteins known to interact with Hsp70. Thus, HopI1 may interfere with protein folding in the chloroplast by interacting with Hsp70 (Jelenska *et al*., 2007). Furthermore, HopI1 can reduce SA levels in plants with constitutively high SA accumulation (Jelenska *et al*., 2007).

Recently, the structure of the type III effector AvrPto1 bound to the Pto kinase was determined (Xing *et al*., 2007). Contrary to what was previously thought, AvrPto1 inhibits Pto's kinase activity. This inhibition is not responsible for initiating Pto/Prf-dependent ETI since mutations that disrupt AvrPto1's inhibition activity retain the ability to elicit a Pto/Prf-dependent HR. These results will likely be used by proponents and opponents of the guard hypothesis. Is Pto an R protein that evolved to look like AvrPto1's real virulence targets or is Pto a virulence target of AvrPto1 that is guarded by Prf? Whatever the answer, this important finding suggests that the ability to inhibit kinases is AvrPto1's virulence activity. It seems that either way Prf is 'guarding' Pto, whether Pto is a bona fide virulence target or a virulence target mimic. Identifying additional targets of AvrPto1 should help to clarify the role of Pto and Prf.

3.5 Influence of type III effectors on plant hormones

Plants have a complex hormonal system to respond to certain types of stresses and developmental cues and bacterial pathogens have evolved strategies to manipulate hormonal regulation during infection (Grant and Lamb, 2006; Robert-Seilaniantz *et al*., 2007). SA is generally associated with resistance to

biotrophic and hemibiotrophic pathogens (including *P. syringae*), jasmonic acid (JA) and ethylene with wounding and necrotrophic pathogens, while abscisic acid (ABA) is associated with abiotic stress (Glazebrook, 2005). *P. syringae* can strongly induce the *Arabidopsis* ethylene response factor *RAP2.6* gene via JA pathways, and several type III effectors have been shown to contribute to its induction (He *et al.*, 2004). Since SA and JA are known to be antagonistic of each other (Kunkel and Brooks, 2002), type III effectors may be acting in part by inducing JA pathways such that plant SA responses are inhibited. Another example of *P. syringae* type III effectors manipulating plant hormones was recently reported (de Torres-Zabala *et al.*, 2007). When plants were treated in this study with wild-type *P. syringae* and compared to those treated with a T3SS defective mutant, genes involved in ABA biosynthesis and signaling were found to be induced by the effectors. Consistent with this result, mutations that rendered plants more sensitive to ABA or exogenous application of ABA allowed increased bacterial growth, while mutants that could not produce ABA showed limited growth of pathogenic and non-pathogenic bacteria (de Torres-Zabala *et al.*, 2007). Notably, overexpression of AvrPtoB in *Arabidopsis* had similar effects on ABA synthesis, indicating that AvrPtoB alters ABA levels in plants (de Torres-Zabala *et al.*, 2007). Recently, *Arabidopsis* plants constitutively expressing the type III effector AvrRpt2 were shown to exhibit phenotypes similar to mutants with altered auxin levels and had increased amounts of the auxin indole acetic acid (Chen *et al.*, 2007). The mechanism by which AvrRpt2 affects auxin levels *in planta* is currently unknown. Clearly, type III effectors can alter hormone levels in plants. More research is needed in this area to define better how hormone imbalances and cross talk between hormone pathways affect pathogenesis and how type III effectors alter hormone levels.

3.6 Concluding remarks

The recent advances of our understanding of activities of *P. syringae* type III effectors have revealed new strategies that bacteria use to invade and multiply in plants. So far, it seems that the primary target for *P. syringae* type III effectors is the plant innate immune system. Research on type III effectors is beginning to deliver on discovery of new components of innate immunity. It may not be possible to identify these components using the currently available genetic and molecular approaches. Apparently, through the course of coevolution *P. syringae* and other plant pathogens have targeted important components of innate immunity – those that when manipulated help the pathogen to grow and cause disease. Thus, to the plant biologist, type III effectors can be viewed as molecular tools to explore plant innate immunity and perhaps other metabolic processes that these pathogens would benefit from by altering. Identifying the targets of type III effectors may lead to crops with improved innate immunity to pathogens through biotechnology.

With recent progress studying effectors from fungal and oomycete pathogens (Birch *et al.*, 2006; Ellis *et al.*, 2007), it will be interesting to see whether they affect the same components of innate immunity that are targeted by type III effectors. One would anticipate that at least some plant proteins targeted by type III effectors would also be targets of eukaryotic effectors since the innate immune responses and outputs are similar regardless of the nature of the pathogen. If this holds to be true, the targets of type III effectors may lead to effective agricultural control strategies against multiple biotic stresses.

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Chapter 4

FUNGAL AND OOMYCETE BIOTROPHY

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Abstract: Biotrophic fungal and oomycete pathogens have strikingly similar mechanisms to invade plants and retrieve nutrients. In particular, the obligate biotrophs form specialised feeding structures such as haustoria and intracellular hyphae that play an important role in host–pathogen signalling and nutrient retrieval. To establish a successful infection, pathogens need to overcome host defences, and suppression of plant defence responses is now a well-known phenomenon in disease. The molecular mechanisms of defence suppression by fungal and oomycete pathogens likely occurs via secreted or translocated effector proteins whose primary function is to establish compatibility. Many fungal and oomycete effector proteins are transported inside the host cell. In interactions with an obligate biotroph, the host provides the necessary nutrients for pathogen growth and reproduction. Specific transporters and metabolic enzymes in both host and pathogen contribute to nutrient exchange between the two organisms and hijacking of the host source–sink balance seems to be important for infection. Studies of plant–biotrophic fungal and plant–oomycete pathogen interactions, now exploiting a wealth of pathogen genome information, are providing unique insights into molecular mechanisms determining disease susceptibility and resistance.

Keywords: haustorium; nutrient transport; source–sink relation; susceptibility; effectors; suppression

4.1 Introduction

Biotrophic fungal and oomycete pathogens share an ability to grow and reproduce on living host tissue. Their filamentous growth and infection mechanisms are strikingly similar, but the evolutionary origin of fungi and

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oomycetes is very different. The majority of phytopathogenic fungi belong to the Ascomycetes and Basidiomycetes, whereas the oomycetes belong to the kingdom Stramenopiles, which also contains the photosynthetic golden-brown algae and diatoms. Genome analysis of two oomycete species, *Phytophthora sojae* and *Phytophthora ramorum*, revealed the presence of genes of probable phototroph origin, suggesting that they have evolved from a photosynthetic ancestor (Tyler *et al.*, 2006). Similarities in growth and infection of fungal and oomycete pathogens have probably arisen through convergent evolution, although there is some evidence for cross-kingdom or lateral gene transfer (Richards *et al.*, 2006).

In this chapter, we focus on the obligate biotrophs that have an absolute dependency on the living host and on a subgroup of hemibiotrophic pathogens that shift from a biotrophic to a necrotrophic stage during infection of the host. Most biotrophs penetrate the host cell wall to form an intimate interaction with host cells by the formation of haustoria or invasive hyphae. Only few species remain exclusively extracellular, such as the imperfect fungal tomato pathogen *Cladosporium fulvum* (Thomma *et al.*, 2005). To infect a given plant species successfully, fungal and oomycete pathogens not only need to overcome mechanical and biochemical barriers and defences, but also have to establish a feeding relationship with the host. We have subdivided the infection process into four distinct steps: (i) the penetration and establishment of infection structures, (ii) the circumvention and suppression of plant defence responses, (iii) the contribution of the host to the infection process and (iv) the acquisition of nutrients from the host. We discuss these different aspects from the viewpoint of the pathogen, and focus on host-related processes since these are of vital importance for obligate biotrophic pathogens.

4.2 Penetration and establishment of infection structures

Obligate biotrophic pathogens typically infect host plants without causing cell death for at least several days. These biotrophs, in contrast to hemibiotrophs and necrotrophs, are characterised by (i) highly developed infection structures, (ii) a narrow contact zone between pathogen and plant plasma membrane, (iii) the formation of specialised feeding hyphae or haustoria and (iv) suppression of host defence responses (Mendgen and Hahn, 2002). As an example, the early infection stages of the oomycete pathogen *Hyaloperonospora parasitica* are depicted in Fig. 4.1.

4.2.1 Spore adhesion

The infection process is initiated when spores adhere to host surfaces and germinate. Although adhesion to plant surfaces is a common theme among pathogens, the mechanisms and compounds used for adhesion are very different between organisms (Dean, 1997). To become securely attached to

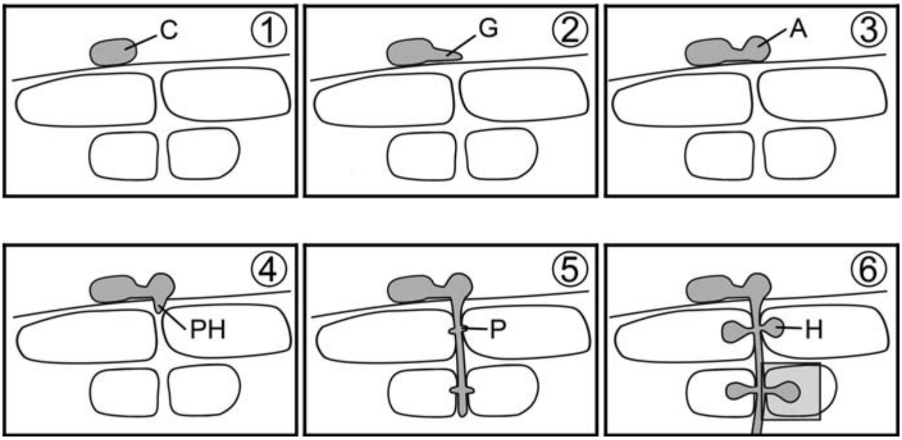

Figure 4.1 Schematic representation of the early infection stages of the haustorium-forming pathogen *Hyaloperonospora parasitica*, the causal agent of downy mildew disease on *Arabidopsis*. (1) A conidiospore (C) lands on the leaf surface and (2) forms a germ tube (G). (3) An appressorium (A) develops at the tip of the germ tube and (4) a penetration hypha (PH) grows through the anticlinal wall between epidermal cells. Plant signals, either chemical or topographical, are thought to provide positional information for the development of appressoria and penetration hyphae. (5) The pathogen grows between plant cells and in almost all cells that the pathogen encounters the formation of haustoria is initiated by local penetration (P) of the plant cell wall. (6) The haustorium (H) invaginates the host cell but remains surrounded by a host-derived membrane, the extrahaustorial membrane, that has an enlarged surface area. A magnification of a haustorium, as depicted in the grey square (6), is further detailed in Figs 4.2 and 4.3.

the host, spores contain mucosal layers and/or secrete adhesives made of heavyweight glycoproteins, lipids and polysaccharides. These layers and components not only aid in adhesion, but are also thought to protect the spores from desiccation, physical damage and host defence responses (Gornhardt *et al.*, 2000). In addition, they can function in perception of physical signals, such as surface hydrophobicity which is important for appressorium formation (Bircher and Hohl, 1997). The oomycete *H. parasitica*, a pathogen of *Brassica* spp., produces two distinct types of extracellular matrices during development (Carzaniga *et al.*, 2001). The first one contains β-1,3-glucans, mannose, *N*-acetylglucosamine and *N*-acetylgalactosamine. This matrix is produced upon surface contact with the host, whereas the second type is produced at the very early stage of germination as a thin film containing various proteins, galactose and *N*-acetylglucosamine. Both of these matrices appear to function in adhesion, but it is likely that these matrices also fulfil additional roles. The chitin wall of appressoria of rust fungi is covered with α- and β-1,3-glucans (Freytag and Mendgen, 1991), which are also found in the walls of germ tubes and appressoria of *Colletotrichum lindemuthianum* (O'Connell *et al.*, 1995). These fungal 1,3-glucans, as in oomycetes, appear to contribute to pathogen attachment (Carzaniga *et al.*, 2001).

4.2.2 Appressorial development

Appressoria are penetration structures that are formed after germination and from which the pathogen enters into the host. Developing appressoria secrete enzymes and/or generate physical force to breach the cuticle and outer cell wall. Surface hardness, hydrophobicity and thigmotropism (i.e. sensing surface topography) are important cues for appressorium development. The amount of pressure a single appressorium can generate is impressive. *Magnaporthe grisea* and probably also *Colletotrichum* spp., such as *Colletotrichum graminicola*, are able to generate pressure higher than 8 MPa (Howard *et al.*, 1991; Bechinger, 1999). This high pressure is obtained by concentrating glycerol in the appressorium, giving an inwards flow of water by osmosis and subsequent turgor generation. In addition, many pathogens also utilise lytic enzymes for cell wall weakening, e.g. *Blumeria graminis* f.sp. *hordei* that utilises cellobiohydrolase I and II (Pryce-Jones *et al.*, 1999).

In general, when an appressorium has developed, a penetration hypha subsequently invades the host tissue, either between epidermal cells or directly by cell penetration. Attempted pathogen entry leads to massive rearrangements of organelles and the actin cytoskeleton as well as to changes in vesicle trafficking and subcellular localisation of host proteins (Takemoto *et al.*, 2003; Koh *et al.*, 2005). Interestingly, while cell integrity is diminished in the host cell, viability seems to remain intact (Lipka and Panstruga, 2005). To give an insight to the large variation in infection strategies, several examples are described below.

Most species in the genus *Colletotrichum* are hemibiotrophs with different time spans of their biotrophic phase. The well-studied anthracnose fungus *C. lindemuthianum* generates a short germ tube after germination of the spore that differentiates into an appressorium. A penetration hypha develops from the appressorium that swells within the epidermal cell to form a vesicle and a broad primary hypha. These structures remain surrounded by a matrix, separating the pathogen from the invaginated host plasma membrane (reviewed by Tucker and Talbot, 2001). A specific fungal glycoprotein, CIH1, could be detected in the matrix only during the biotrophic interaction (Perfect *et al.*, 1998). The regulated expression of pathogen genes and proteins during the biotrophic stage supports the idea that this stage of infection requires specific adaptations of the pathogen. The biotrophic relationship ends when narrow secondary hyphae develop, the plant plasma membrane starts to disintegrate and massive amounts of degrading enzymes are released.

The obligate biotroph *Uromyces fabae* follows a similar route at the early stages of infection by forming a germ tube from a spore that is attached to the host via a so-called adhesion pad. The germ tube is able to recognise the physical features of the stomatal pore on which an appressorium is formed. From this, a penetration hypha grows and develops into an infection hypha. When the tip of the infection hypha contacts a mesophyll cell, a haustorial mother

cell is formed. The haustorial mother cell invades a mesophyll cell in which a haustorium develops. After formation of the first haustorium, branching of infection hypha takes place and more intercellular hyphae, haustorial mother cells and haustoria are being formed (Mendgen and Hahn, 2002).

The powdery mildews, e.g. *B. graminis*, infect only the epidermal layers of their respective hosts. After a spore lands on the host surface, one or two germ tubes emerge from it, depending on the genus. The germ tube swells into an appressorium and directly penetrates the host cell wall using a combination of lytic enzymes and mechanical power. After entering, powdery mildew develops a haustorium by invagination of the plant plasma membrane (Huckelhoven, 2005).

4.2.3 The haustorium

Host entry and the early stages of infection are remarkably similar between the species described above, as they all involve spore adhesion, germination and penetration by appressoria. After all, these pathogens have to overcome similar chemical and physical barriers. Later infection stages are quite different. Hemibiotrophs, like *C. lindemuthianum*, shift to the nectrophic phase and develop only intracellular hyphae, which tend to be less specialised infection structures than haustoria (Perfect and Green, 2001). Obligate biotrophs are characterised by the formation of haustoria. Haustoria and intracellular hyphae not only are involved in the acquisition of water and nutrients, but have also been implicated in signalling, communication and avoidance of host defence responses (Mendgen and Deising, 1993). There is still debate about the advantage of haustoria over intercellular hyphae. It seems that in the exclusively extracellular fungal pathogens *C. fulvum* and *Septoria tritici*, nutrients in the apoplast are sufficient to support their growth (Spencer-Philips, 1997). Haustoria and intracellular hyphae, on the other hand, are in close contact to host cells which might allow them a easier access to the intracellular pool of nutrients. The observation that haustoria mainly develop in obligate biotrophic interactions could reflect their adaptation to a prolonged association with their hosts. Haustorium formation in vitro has recently been described for *U. fabae*. Following in vitro spore germination and appressorium formation, the addition of volatile aldehydes, which are known to be involved in plant defence, induced haustorium formation, albeit on a limited scale. Haustorium formation in vitro and in vivo was enhanced by decanal, nonanal and hexenyl acetate, whereas it was reduced by terpenoid farnesyl acetate (Mendgen *et al.*, 2006). This suggests that specific host chemicals serve as cues for pathogen development. Biotrophic pathogens may require a balanced mix of chemicals and other cues to develop and grow. In vitro these conditions may not be easily met, making these organisms highly dependent on their hosts for growth and reproduction.

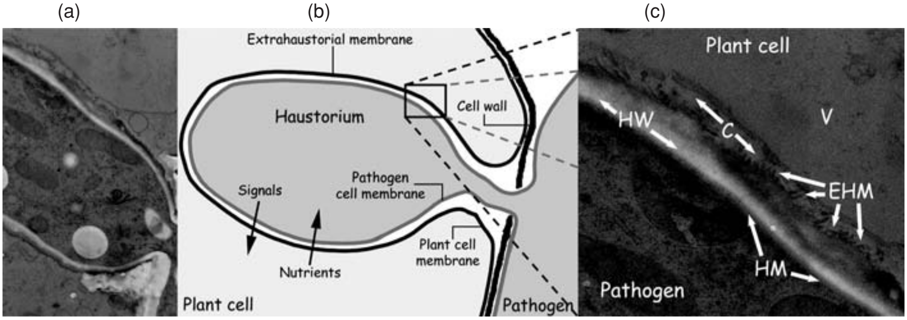

Figure 4.2 Electron micrograph and schematic representation of an haustorium in the *Arabidopsis–Hyaloperonospora parasitica* interaction. (a) Electron micrograph of part of a *H. parasitica* haustorium in an *Arabidopsis* cell. The pathogen has breached the plant cell wall (lower right-hand side) and an haustorium has invaginated the host cell. The haustorium contains organelles, e.g. mitochondria, vacuoles, golgi and nuclei. (b) Schematic representation of a haustorium. The extrahaustorial membrane (EHM) is the place where the exchange of signals and nutrients occurs. The plant cell membrane is continuous with the EHM. (c) Enlargement of a section of the EHM. The pathogen is separated from the plant cell cytoplasm by a haustorial membrane, haustorial wall and the EHM. The EHM exhibits a ruffled structure which is not observed in normal host plasma membranes. A thin layer of cytosol (C) resides between the EHM and the vacuole (V) of the plant cell.

4.2.4 The extrahaustorial membrane

The membrane surrounding the haustorium is known as the extrahaustorial membrane (EHM) and is continuous with the plant plasma membrane. Striking morphological differences between the EHM and the plant plasma membrane are the lack of intramembrane particles and a ruffled structure (Mims *et al.*, 2004). This is thought to improve nutrient acquisition of the pathogen by an increase in surface area (Harder and Mendgen, 1982). The ruffling of the EHM is depicted in electron micrographs of *Arabidopsis* cells infected with *H. parasitica* (Fig. 4.2).

The EHM of powdery mildew was shown to be deprived of at least a subset of plant plasma membrane proteins. This was demonstrated using transgenic lines containing GFP (green fluorescent protein)-tagged plasma membrane proteins that normally localise to the host plasma membrane (Cutler *et al.*, 2000; Koh *et al.*, 2005). In these studies, a boundary was observed between the EHM and the host plasma membrane. This so-called neckband is found in many haustoria, although not in downy mildews, and likely seals the extrahaustorial matrix from the host apoplast (Szabo and Bushnell, 2001). The invaginated cell membrane surrounding intracellular hyphae is much less specialised than the EHM and appears to be more similar to the plant plasma membrane (O'Connell, 1987).

A particular interesting aspect of haustoria is the interface between these structures and the host, termed the haustorial matrix or wall. The origin of the

matrix is not clear, although there is evidence for both host- and pathogen-derived carbohydrates and glycoproteins. In the *Vigna sinesis–Uromyces vignae* interaction, host-derived secretion of matrix components has been described (Stark-Urnau and Mendgen, 1995). Secreted fungal glycoproteins were shown to specifically reside in the haustorial matrix by using monoclonal antibodies. These proteins are not present in other walls of hyphae and appressoria, arguing for a distinct function of haustoria (Mackie *et al.*, 1993; Murdoch *et al.*, 1998). These findings show that the haustorium is truly a remarkable structure with its own specialised function in biotrophy.

4.3 Dealing with plant defence responses

To establish successful infections, pathogens have to deal with a wide array of defence responses of the host. Basal defence of plants is triggered by non-specific elicitors or pathogen-associated molecular patterns (PAMPs), named PAMP-triggered immunity (PTI) (Chapter 2). Pathogens have evolved effectors to aid the infection process, e.g., by acting as suppressors of PTI (Chisholm *et al.*, 2006; Jones and Dangl, 2006). In turn, plants have evolved to recognise these effectors, directly or indirectly, allowing them to trigger defence responses that arrest pathogen ingress. This effector-triggered immunity is again often overcome by pathogens through mutation or deletion of effectors. This evolutionary model of plant immunity is nicely described in the zigzag model (Jones and Dangl, 2006).

PTI, such as non-host resistance against non-adapted pathogens, is triggered by pathogen recognition and subsequent activation of plant defence responses. In *Arabidopsis*, it was found that resistance against the non-adapted powdery mildews from grass and pea could be subdivided into a distinct pre- and post-invasion phase (Lipka *et al.*, 2005). The pre-invasion defence was shown to be dependent on the glycosyl hydrolase PEN2, which might be involved in the production of an antimicrobial compound that blocks penetration (Chapter 8). The post-invasion defence required EDS1/PAD4/SAG101, which form protein complexes that are involved in *R* gene-mediated and basal resistance (Feys *et al.*, 2005).

4.3.1 Suppression of PTI

Adapted pathogens have evolved ways to circumvent or suppress PTI, thereby escaping the intrinsic defence capacity of their hosts. Evidence for active suppression of defence was already provided more than 30 years ago by Ouchi *et al.* (1974), who observed induced susceptibility in host cells that were infected with virulent powdery mildew isolates. Subsequent challenge with an isolate that is normally non-virulent (avirulent) resulted in successful penetration and pathogen growth. This phenomenon is also referred to

as induced accessibility (Ouchi *et al.*, 1974) and has since been observed in other interactions, e.g. the suppression of *Mla1*-mediated resistance in barley by virulent *B. graminis* strains (Lyngkjaer *et al.*, 2001) and the induced susceptibility of *Brassica juncea* to *H. parasitica* after pre-inoculation with the oomycete *Albugo candida* (Singh *et al.*, 2002). The discovery that fungal and oomycete effector proteins act inside the host cell, as described further on in this chapter, has opened the way to study the molecular mechanisms by which these proteins suppress defence responses and induce accessibility.

A limited number of examples of effector-mediated suppression have been described. In *Phytophthora infestans*, infection is characterised by an initial biotrophic phase in which the pathogen does not trigger cell death and the *Avr3A* gene was shown to suppress the cell death response that is triggered by the elicitor INF1 (Bos *et al.*, 2006). Only at later stages of infection with *P. infestans*, during the necrotrophic phase, are host cells killed. Suppression of defence or induction of susceptibility was also observed by transient expression *in planta* of the powdery mildew effectors *Avr-a10* and *Avr-k1* (Ridout *et al.*, 2006). In single cell biolistics assays, it was found that overexpression of *Avr-a10* and *Avr-k1* resulted in an enhanced infection success on susceptible host plants.

Other forms of suppression or circumvention are targeted to extracellular plant defence responses that involve antimicrobial proteins and products, i.e. PR proteins (van Loon *et al.*, 2006). Diverse mechanisms of protection against these antimicrobial activities have evolved. Research on the extracellular proteins of the fungal tomato pathogen *C. fulvum* has revealed several interesting mechanisms of protection (Thomma *et al.*, 2005). The secreted Avr4 protein has a conserved pattern of four disulphide bridges resembling an invertebrate chitin-binding domain (van den Burg *et al.*, 2004). Indeed, experimental evidence was obtained that Avr4 binds chitin, which is part of the fungal cell wall and is able to protect the polymer from degradation by plant chitinases that are produced during plant defence (van den Burg *et al.*, 2006). Another cysteine-rich secreted protein of *C. fulvum*, Avr2, inhibits the activity of the tomato cysteine protease Rcr3 which is thought to have an, as yet unknown, antimicrobial activity (Rooney *et al.*, 2005). In resistant tomato plants, the *Cf-2*-encoded protein guards Rcr3 and responds to alterations that might disturb its function, e.g. the binding of Avr2 (Kruger *et al.*, 2002).

Oomycetes also have evolved protease inhibitors to counteract proteases that are secreted into the apoplast as part of the plant defence response. Two Kazal-like protease inhibitors of *P. infestans*, EPI1 and EPI10, interact with and inhibit the pathogenesis-related tomato subtilisin-like serine protease P69B (Tian *et al.*, 2004, 2005). Other protease inhibitors were identified in *P. infestans*, known as EPICs, that are secreted proteins with a cystatin-like protease inhibitor domain (Tian *et al.*, 2007). EPIC2B was shown to interact with a protease that is closely related to tomato Rcr3.

4.3.2 Effectors triggering immunity

Pathogen effectors that interfere with host cell processes, e.g. the suppression of PTI, are the types of proteins that are recognised directly or indirectly by plant resistance genes. In recent years, it was discovered that several proteins of biotrophic fungal and oomycete pathogens that trigger effector-triggered immunity are being recognised inside the host cell (see Table 4.1).

These proteins are secreted by the pathogen and must enter host cells to induce *R* gene-mediated resistance as their recognition is shown to occur in the host cell cytoplasm. However, the mechanism by which these effectors gain access to host cells remains unclear. All effectors that have been identified so far are directly or indirectly recognised by cognate R proteins that are predicted to be localised in the host cell cytoplasm. Specific recognition has, in most cases, been unequivocally assessed by using near-isogenic plant lines that possess or lack the corresponding *R* gene. In Table 4.1, an overview

Table 4.1 Fungal and oomycete effector proteins that act inside the host cell to trigger *R* gene-mediated resistance

***Avr* gene**	**Pathogen**	**Signal peptide**	**Host *R* gene**	**Proof**	**Reference**
Avr1b	*Phytophthora sojae*	Yes	Soybean *Rps1b*	Protein infiltration	Shan *et al.* (2004)
Avr3a	*Phytophthora infestans*	Yes	Potato *R3a*	Biolistics and transient *A. tum*	Armstrong *et al.* (2005)
ATR13	*Hyaloperonospora parasitica*	Yes	*Arabidopsis RPP13*	Biolistics	Allen *et al.* (2004)
ATR1	*Hyaloperonospora parasitica*	Yes	*Arabidopsis RPP1*	Biolistics	Rehmany *et al.* (2005)
AvrL567	*Melampsora lini*	Yes	Flax *L5, L6, L7*	Stable and transient *A. tum*	Dodds *et al.* (2004)
AvrM(-A)	*Melampsora lini*	Yes	Flax *M*	Transient *A. tum*	Catanzariti *et al.* (2006)
AvrP4	*Melampsora lini*	Yes	Flax *P4*	Transient *A. tum*	Catanzariti *et al.* (2006)
AvrP123-A	*Melampsora lini*	Yes	Flax *P1, P2*	Transient *A. tum*	Catanzariti *et al.* (2006)
Avr-a10	*Blumeria graminis*	No	Barley *Mla10*	Biolistics	Ridout *et al.* 2006
Avr-k1	*Blumeria graminis*	No	Barley *Mlk1*	Biolistics	Ridout *et al.* (2006)
Avr-Pita	*Magnaporthe oryzae*	Yes	Rice *Pita*	Biolistics	Jia *et al.* (2000)

is given of known effector proteins, the cognate *R* genes that mediate their recognition and the method by which their activity inside host cells has been demonstrated.

Four oomycete effectors, Avr1b, Avr3a, ATR13 and ATR1A, share a common motif, the RXLR sequence (Armstrong *et al.*, 2005; Rehmany *et al.*, 2005) that appears to act as a host-targeting signal (Bhattacharjee *et al.*, 2006). The RXLR motif is located within 60 amino acids of the signal peptide (SP) sequence that mediates the ER-type of secretion and is in many cases followed by a motif further downstream that is enriched in aspartic and glutamic acid residues. The RXLR motif resembles the RXLX motif that has been described as a host-targeting signal in the malaria parasite *Plasmodium falciparum* and is even functionally conserved, as demonstrated by translocation of an AVR3a-GFP fusion protein from *P. falciparum* into erythrocyte host cells (Bhattacharjee *et al.*, 2006). Recent studies on Avr3a revealed that both the RXLR and the EER motifs are required for translocation into host plant cells. These motifs are, however, not involved in the secretion of the protein from haustoria (Whisson *et al.*, 2007).

Of the seven fungal effector proteins that have been described to act as avirulence proteins, four were identified in *Melampsora lini*, the flax rust pathogen. The *M. lini* Avr proteins are predicted to be secreted from the pathogen as they carry a typical N-terminal SP. However, there is no common or known motif in the Avr proteins that could explain how they are transferred into host cells. This is also the case for another rust candidate effector from *U. fabae*, RTP1p, which carries an SP and was found to be secreted from haustoria and transferred into the host cell nucleus (Kemen *et al.*, 2005). Using a polyclonal antibody against RTP1p, the glycosylated protein could be detected in the extrahaustorial matrix and in the cytoplasm and nucleus of host cells that contained a fungal haustorium but not in uninfected cells. A last example of an SP-containing effector is the Avr-Pita protein from the hemibiotroph *Magnaporthe oryzae* (Jia *et al.*, 2000). At early stages of infection, *M. oryzae* penetrates host cells by invasive hyphae that remain surrounded by a plant-derived membrane, the so-called extra-invasive hyphal membrane (Kankanala *et al.*, 2007). Avr-Pita is secreted by the invasive hyphae (Kankanala *et al.*, 2007) and has to cross the extra-invasive hyphal membrane in order to reach the host cell cytoplasm where it physically interacts with the resistance protein Pita (Jia *et al.*, 2000). For two powdery mildew effector proteins, Avr-a10 and Avr-k1, it is unclear how they are secreted from the pathogen. Both proteins lack a typical SP for secretion through the ER, but could be transported via an alternative, non-conventional secretion route (Ridout *et al.*, 2006). The finding that fungal effectors that act inside the host cell do not necessarily possess a typical SP for secretion is important to consider when searching for candidate effector proteins in sequenced genomes, as there is a strong tendency to focus exclusively on SP-containing proteins.

The challenge for the future is to (i) identify all effector proteins encoded by the genomes of biotrophic fungal and oomycete pathogens, and more

importantly, (ii) unravel their molecular function in the host cell. Bioinformatics approaches have resulted in the identification of several hundred candidate RXLR effector proteins in oomycetes (Whisson *et al.*, 2007; Win *et al.*, 2007). Strikingly, there is only a small overlap between the predicted RXLR effector candidates of *P. sojae*, *P. ramorum* and *H. parasitica*, suggesting that they have undergone accelerated evolution. The identification in fungal pathogens is more complex, as there is no clear signature that different effectors have in common. In the genome of the recently sequenced basidiomycete pathogen *Ustilago maydis*, a large number of 426 proteins were predicted to be secreted (Kamper *et al.*, 2006). For 298 (70%) of these, a function cannot be ascribed, and 193 are specific for *U. maydis*. Several of these proteins are candidate effectors and could be transported over a host-derived membrane that surrounds the intracellular hyphae during biotrophic growth of *U. maydis*.

4.4 Contribution of the host to infection

The modulation of plant defence responses by active suppression is an important process that biotrophic pathogens use to establish compatibility. Besides dealing with plant defences, biotrophs are believed to actively interfere with host cell processes that are related to the development of infection structures, the establishment of a feeding relationship and interference with the physiology of the plant. Although this seems logical and clear, our understanding of the modulation of host cell processes by fungi and oomycetes is still very limited. To understand the contribution of the host to the infection process and identify plant genes that aid the pathogen in establishing basic compatibility, different genetic approaches have been taken: (i) cloning of recessive resistance genes, (ii) mutagenesis and (iii) candidate gene approaches.

4.4.1 Recessive resistance genes

These genes can be viewed as host genes that in their intact form are essential for pathogen growth, but in their defective form (as a result of deletion or mutation) do not pose a problem for the normal functioning of the host. In contrast to the resistance conferred by dominant *R* genes, knowledge is limited on recessive resistance genes. Recessive forms of resistance have been identified in many plant–pathogen interactions including plant–virus, plant–bacteria, plant–fungus or plant–oomycete interactions. In barley plants homozygous for the recessive *mlo* gene, the entry of powdery mildew (*B. graminis* f.sp. *hordei*) into epidermal cells is blocked. *Mlo* encodes a plasma membrane protein with seven transmembrane domains (Buschges *et al.*, 1997) that is required for successful penetration of the host cell wall (Panstruga, 2005). In *Arabidopsis*, 15 *Mlo* orthologues were identified. To test if the *Mlo* orthologues confer resistance when mutated, homozygous T-DNA lines were generated and challenged with the powdery mildew fungus *Golovinomyces orontii*. Only

the homozygous *Atmlo2* line showed strongly reduced disease symptoms and conidiophore formation (Consonni *et al.*, 2006). *AtMLO2* belongs to a clade of three *Mlo* orthologues, including *AtMLO6* and *AtMLO12*. Double mutants of *Atmlo2/Atmlo6* and *Atmlo2/Atmlo12* supported lower levels of fungal growth than the single *Atmlo2* mutant and the *Atmlo2 Atmlo6 Atmlo12* triple mutant was fully resistant, similar to barley *mlo* plants (Consonni *et al.*, 2006). It is unclear at this point if in susceptible plants the MLO protein is recruited by the powdery mildew pathogen to aid the infection process, or if the wild-type MLO protein acts solely as a negative regulator of defence. Knowledge on the molecular basis of other forms of recessive resistance to fungal and oomycete pathogens is still lacking, except for those forms that have been generated by mutagenesis, as discussed below.

4.4.2 Susceptibility genes identified by mutagenesis

Loss-of-function alleles of dominant susceptibility genes leading to recessive forms of resistance can be generated by mutagenesis. This approach was pioneered by Vogel and Somerville (2000), who isolated 26 *powdery mildew resistant* (*pmr*) mutants in *Arabidopsis* that had lost susceptibility to the powdery mildew pathogen *Erysiphe cichoracearum*. Four of the corresponding *PMR* genes have been cloned so far. *PMR4* (*GLS5=CalS12*) encodes a callose synthase, resulting in a loss of callose accumulation. Gene expression profiling of *pmr4-1* plants showed an enhanced activation of salicylic acid- and pathogen-responsive genes. The upregulation of these genes in *pmr4-1* mutants increased even further in response to pathogen infection. The resistance mediated by *pmr4* probably acts through both *NPR1*-dependent and *NPR1*-independent signalling pathways since the *pmr4 npr1* double mutant still supported an intermediate level of fungal growth (Nishimura *et al.*, 2003). *PMR6* encodes a pectate lyase-like gene, and *PMR5* is a gene of unknown function belonging to a large family of plant-specific genes (Vogel *et al.*, 2002, 2004). The cell wall composition of *pmr6* is altered and analysis of *pmr5* indicates similar changes, i.e. increased levels of pectin. *PMR2* was found to be identical to *AtMLO2*, which is described above as an orthologue of the barley *Mlo* gene (Consonni *et al.*, 2006). The PMR proteins are suggested to participate at diverse locations near the haustorial interaction site and could possibly be recruited by the powdery mildew pathogen to establish a successful infection. Absence of any of these PMR proteins would then impair the establishment of compatibility and render the plant resistant.

Other screens in *Arabidopsis* for loss of susceptibility to fungal and oomycete pathogens have been performed. Three candidate *chr* mutants have been isolated that show resistance to the hemibiotrophic fungal pathogen *Colletotrichum higginsianum* (O'Connell and Panstruga, 2006). In our laboratory, we have identified six *Arabidopsis* loci, generated by ethyl methane sulfonate-mutagenesis, that confer recessive resistance to the downy mildew pathogen *H. parasitica* (Van Damme *et al.*, 2005). Three of the *downy mildew resistant*

(*dmr*) mutants, *dmr3*, *dmr4* and *dmr5*, show enhanced defence responses in the absence of the pathogen and are likely mutated in a negative regulator of plant defence. By contrast, the *dmr1*, *dmr2* and *dmr6* mutants do not show high levels of expression of the defence-associated *PR-1* gene. They are also more specifically impaired in the interaction with *H. parasitica* and are not resistant to the bacterial pathogen *Pseudomonas syringae*. *DMR1* has been identified as homoserine kinase, mutation of which leads to *in planta* accumulation of homoserine (Van Damme and van den Ackerveken, unpublished data). Exogenous application of homoserine to *Arabidopsis* seedlings induces resistance to *H. parasitica*, suggesting that an unknown mechanism of resistance is triggered by this compound. *DMR6* was identified as a gene encoding a 2OG-Fe(II) oxygenase of unknown function (Van Damme *et al.*, 2008). Strikingly, the *DMR6* gene is transcriptionally activated during plant defence and during a compatible interaction with *H. parasitica*, suggesting a defence-associated role. However, mutation of the gene leads to downy mildew resistance. Resistance mediated by *dmr6* could either be caused by the lack of a metabolic product that is required to support the infection process or alternatively the DMR6 substrate accumulates and triggers defence responses that arrest downy mildew growth.

4.4.3 Candidate gene approach

A third method to identify host genes involved in supporting the infection process is the use of candidate genes that have been identified in other biological systems. An example of such an approach has been described for *Lotus japonicus* (Mellersh and Parniske, 2006). Genetic studies on mutualistic interactions between *Lotus* and microbial symbionts resulted in the identification of a number of common symbiosis (*SYM*) genes (Kistner *et al.*, 2005). The *sym* mutants are impaired in establishing an effective symbiosis with nitrogen-fixing rhizobacteria as well as with mycorrhiza fungi that normally form arbuscular-type haustoria in root cells. Although clearly defective in the interaction with the symbionts, the *sym* mutants did not show a reduction in susceptibility to the rust pathogen *Uromyces loti* (Mellersh and Parniske, 2006). This suggests that there is no significant overlap between the elements needed to establish a compatible interaction with symbionts or pathogens. However, it might be that the main difference is caused by the tissues analysed since the symbionts infect the root whereas the rust infects the *Lotus* leaves.

4.5 Acquiring nutrients from the host

Obligate fungal and oomycete pathogens make intimate contact with the host to aid the acquisition of nutrients for growth and reproduction. This is why, over the years, haustoria have been described as feeding structures.

There are two main hypotheses to explain why obligate biotrophs are fully dependent on the living host for their survival. Either (i) they are incapable of synthesising certain metabolites and require the host to provide them with these compounds or (ii) they are fully prototrophic but require the living host to provide cues for their development and growth within the plant. So far, there is no good evidence to support the first hypothesis. Expressed sequence tag sequencing of obligate biotrophs, e.g. of *B. graminis*, did not reveal the lack of particular metabolic enzymes (Thomas *et al.*, 2001; Spanu, 2006). The availability of the complete genome sequences of downy and powdery mildew pathogens will allow a genome wide comparison with the genomes of related pathogens that can also grow saprophytically. Many groups have tried to cultivate obligate biotrophs in vitro by the addition of plant extracts and compounds to culture media, but so far this has been without success. The second hypothesis of specific cues of plant origin that are required for pathogen development is more likely, although solid evidence is lacking.

Although obligate biotrophs do not appear to be auxotrophic, they utilise plant tissues as their only substrates for growth and reproduction. Thus, they need to establish a feeding relation with their hosts. There are obviously many differences in the modes of nutrient retrieval between fungal and oomycete biotrophs since they have independently evolved a pathogenic lifestyle. In the next paragraph, we describe our current knowledge of a well-studied system with respect to nutrient transport, the interaction between *Vicia faba* (broad bean) and the rust fungus *U. fabae*. Information from other host pathosystems is also discussed.

4.5.1 Nutrient transport in the *Uromyces* system

Detailed analyses of the *V. faba–U. fabae* interaction revealed that hexose (*Uf-HXT1*) and amino acid transporters (*Uf-AAT2*) are highly expressed in haustoria (Hahn *et al.*, 1997; Voegele *et al.*, 2001). Functional characterisation of *Uf*-HXT1p showed a high affinity for D-glucose and D-fructose (Voegele *et al.*, 2001) whereas no function for *Uf*-AAT2p is described. *Uf*-AAT1p, which is a close homologue of *Uf*-AAT2p, has a specificity for L-lysine and L-histidine (Struck *et al.*, 2002), but its expression is not confined to haustoria. β-Glucosidase 1 (*Uf*-BGL1p) is also localised to haustoria and can utilise cellobiose as substrate and could supply *Uf*-HXT1p with glucose since it is targeted to the secretory pathway of *U. fabae* (Haerter and Voegele, 2004). Furthermore, a highly expressed symporter H^+-ATPase (*Uf-PMA1*) was found in haustoria (Struck *et al.*, 1998), which could provide the proton gradient for nutrient transport. In addition, two abundant components, *Uf-THI1* and *Uf-THI2*, of the vitamin B1 (thiamine pyrophosphate) biosynthetic pathway were shown to localise exclusively to haustoria (Sohn *et al.*, 2000). Recent work on *U. fabae* suggests an important role for mannitol in haustoria. Mannitol cannot be produced or metabolised by *V. faba*, but it is thought to protect *U. fabae*

by scavenging reactive oxygen species. Mannitol accumulation is accompanied by high expression of a mannitol dehydrogenase (*Uf*-MAD1p) in haustoria, which could produce mannitol from plant-derived fructose (Voegele *et al.*, 2005). In addition, D-arabitol dehydrogenase (*Uf*-ARD1p), which localises to the haustorial lumen, most likely produces D-arabitol. Arabitol, like mannitol, could act as a radical scavenger, but could also function in carbohydrate storage (Link *et al.*, 2005). Conversion of *in planta* carbohydrates into polyols such as mannitol could switch the haustoria-containing cells into sink tissues or enhance their sink strength, generating a constant influx of sugars and/or other metabolites. Interestingly, there is also an invertase present at the periphery of the rust haustorium, *Uf*-INV1p, that has high affinity for sucrose (Voegele *et al.*, 2006). The contribution of this invertase to pathogenicity needs to be determined, but one can envision that it enhances the sink strength of the infected tissue. These studies on *Uromyces* clearly show that there are specific transporters in the haustorial membrane and enzymes in and around the haustorium, consistent with it being a prime site for nutrient uptake.

4.5.2 Source versus sink tissues

When examining plant sugar metabolism, tissues can broadly be divided into source and sink. Source tissues produce an excess of sugars and other metabolites that can be transported via the phloem to tissues not able to synthesise sufficient amounts themselves or that are used for storage purposes, e.g. seeds, fruits and tubers (Sonnewald and Willmitzer, 1992). Source-to-sink interactions can be regulated at three different levels: assimilate (i) production, (ii) utilisation and (iii) allocation. The first two options are obvious, as higher metabolite production enhances the source strength whereas higher utilisation raises the strength of the sink. Allocation is the process in which proteins mediate transport of nutrients that then determine the role and strength of the tissue. For example, when the production of metabolites remains similar but the number of sugar uptake transporters in the sink is elevated, the strength of the sink is enhanced. In addition, proteins like synthases and invertases can convert metabolites, thereby contributing to the sink strength (Sonnewald and Willmitzer, 1992). Before considering the importance of source-to-sink interactions in biotrophic interactions, a brief overview of key components involved is given. Although many metabolites can be transported, sucrose transport is considered here as a model for source-to-sink relations as sucrose is by far the dominant form of soluble carbon in *Arabidopsis* and many other plant species (Haritatos *et al.*, 2000). Sucrose can move passively through plasmodesmata or, in the case of isolated cells or tissue, is released into the apoplast. Sucrose from source cells is transported into companion cells which are connected via plasmodesmata to sieve elements. The phloem then transports the sucrose to sink cells by either symplastic unloading via plasmodesmata or apoplastically via sugar transporters (Stadler *et al.*, 2005a,b). In apoplastic sinks, like guard cells and other cell types, sucrose can be split

by secreted invertases into glucose and fructose. Sinks can translocate these products by monosaccharide transporters or sucrose itself can be taken up by specific sucrose transporters. Interestingly, during seed development of *V. faba*, a monosaccharide transporter (*Vf-STP1*) and a sucrose transporter (*Vf-SUT1*) are expressed at different developmental stages. These transporters are spatially expressed on the basis of the quality of the apoplastic sinks (Weber *et al.*, 1997). Sink-specific expression of sucrose transporters is observed in various other plants (Sauer, 2007), arguing for a general source–sink mechanism in many, if not all, plant species.

The source-to-sink transition not only is important in the physiology of the plant, but likely also plays an important role in plant–biotroph interactions. The identified components in the *U. fabae* haustorium and periphery support the idea that haustoria are important structures in nutrient retrieval and utilisation and in increasing the sink strength of infected host cells. As biotrophs obtain metabolites from the host, they compete with sink tissue for the available metabolite pool (Fig. 4.3). Pathogens could convert host tissue into 'sink' holes, as many pathogen genes encoding nutrient uptake proteins, e.g. genes involved in transport of amino acids, fatty acids, nitrogen and sugars, are upregulated during plant interactions (Divon and Fluhr, 2007).

4.5.3 Induction of infection-related metabolic genes

Characterisation of *in planta* induced *U. fabae* genes from a haustorium-specific cDNA library by microarray analysis identified a plethora of genes which potentially localise to haustoria and function in metabolic processes and nutrient transport (Jakupovic *et al.*, 2006). However, many of those genes have no similarity to genes in publicly available database and their particular role in pathogenicity remains to be elucidated. A similar approach with cDNA microarrays from *B. graminis* f.sp. *hordei* (barley powdery mildew) revealed that during appressoria and haustoria formation transcripts encoding glycolytic enzymes were greatly induced (Both *et al.*, 2005). This is in accordance with the observation that glucose is the predominant carbohydrate source taken up by *B. graminis* (Clark and Hall, 1998; Sutton *et al.*, 1999). Work on the model plant *Arabidopsis* has shown that both glucose uptake and invertase activity are increased after powdery mildew inoculation. Two *Arabidopsis* transcripts, *At-STP4* and *At-βfruct1*, were found to be induced upon infection with the powdery mildew fungus *E. cichoracearum*. Enhanced glucose uptake in host tissue was observed that coincided with the induction of the sucrose transporter 4 (*AtSTP4*), supporting the 'sink' theory. The cell wall invertase *At-βfruct1* could elevate levels of soluble carbohydrates such as hexoses available to the pathogen (Fotopoulos *et al.*, 2003). In the *Albugo candida* (white blister rust)–*Arabidopsis thaliana* interaction, an increase in invertase activity and carbohydrate accumulation was also observed (Chou *et al.*, 2000). A recent study in wheat showed that glucose uptake is also enhanced after infection with *B. graminis*. Using uncouplers, it was established

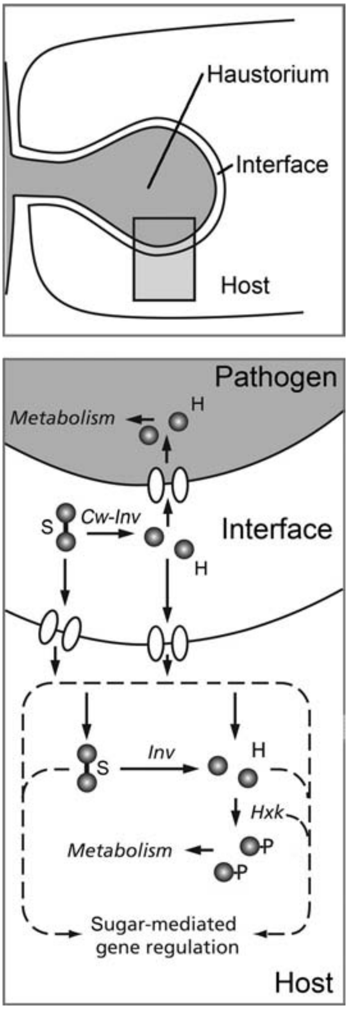

Figure 4.3 Schematic representation of sugar transport and metabolism at the host–pathogen interface. The interface between host and pathogen, e.g. at the haustorium, is the major site of nutrient transport between the two organisms. Extracellular sugars are normally taken up by the host by transporters in the plant cell membrane. The host adapts its gene-regulation and metabolism in response to sugar levels through various sugar-sensing mechanisms. Cleavage of sucrose (S) by invertases (*Inv*) into hexoses (H) and subsequent activation by hexokinases (*Hxk*) provides host cells with metabolic precursors. In infected host cells, the haustorium competes for the extracellular pool of sugars. Extracellular sucrose (S) can be cleaved into hexoses (H) via a cell wall-bound invertase (*Cw-Inv*). This invertase could be of pathogen origin or be actively induced in the host during the infection process. These hexoses can be taken up by the pathogen to be metabolised. As a result, the pathogen alters the sugar status of infected cells, thereby enhancing their sink strengths. The proposed mechanism of sugar transport and metabolism during plant–pathogen interactions is adapted from Biemelt and Sonnewald (2006).

that this uptake is energy-dependent, arguing for similar H^+-ATPase activity in *B. graminis* as in *U. fabae* (Sutton *et al.*, 2007). Measuring the *Triticum aestivum* (wheat) invertase activity after infection also showed an increase in invertase activity (Sutton *et al.*, 2007). Similarly, a higher activity of cell wall-bound invertase was observed in barley challenged with powdery mildew (Swarbrick *et al.*, 2006). Enhanced activities of enzymes responsible for sucrose transport and degradation lower the sugar status of infected tissue. This is observed not only by the increased carbohydrate transport to, e.g., *B. graminis* and enzymes involved in this process, but also by lowered starch levels (Storr and Hall, 1992; Heisteruber *et al.*, 1994). Powdery mildew infects only epidermal cells that do not photosynthesise. Strikingly, while the cells surrounding infection sites remain healthy green colour ('the green island effect'), other cells become chlorotic. This shows that infection suppresses cell death and that the green cells are maintained viable as a nutrient source (Huckelhoven, 2005). This argues for the idea that powdery mildews, and likely many other obligate biotrophs such as rusts, generate an alien 'sink' hole in the host, causing a shift in carbohydrate partitioning. This shift provides the biotroph with an ample supply of carbohydrates, allowing it to grow and complete its life cycle.

At present, little information is available on the transport and metabolic events taking place in hyphae and haustoria of obligate oomycete plant pathogens. For example, several studies report on *H. parasitica* genes that are expressed *in planta* in *A. thaliana* (van der Biezen *et al.*, 2000; Bittner-Eddy *et al.*, 2003). However, no in situ information and functional analyses are available on (putative) transporters and enzymes. Therefore, it remains speculative whether these oomycete proteins have analogous functions to their fungal counterparts. Nevertheless, based on phenotypic similarities, it is likely that similar mechanisms of nutrient transport operate in obligate oomycete pathogens as in powdery mildews and rusts.

4.6 Outlook

The study of plant–biotrophic fungal and plant–oomycete pathogen interactions is providing a unique insight into the molecular mechanisms that determine disease susceptibility. Although research is scattered over many different host–pathogen systems, several powerful model systems are emerging. In addition, genome sequencing is providing us with a wealth of information on the genes and proteins that are involved in pathogenicity and the interaction with the plant. The field of pathogen comparative genomics will allow us to study gene evolution and the identification of fast-evolving and species-specific genes that are the prime candidates for being involved in adaptation to the host. The vast number of potential effector proteins that are active in the host cell is a superb resource for future studies aimed at understanding the molecular basis of plant disease susceptibility. The obligate biotrophic rusts,

powdery mildews and downy mildews are ideal systems to identify effectors that can then be studied in host plants that are amenable to functional studies, e.g. *Arabidopsis*, flax, *Medicago*, *Lotus*, rice and barley. However, functional studies on obligate biotrophs are hampered by the lack of transformation and gene knockout methods. Hemibiotrophic pathogens offer the possibility to study functional aspects of biotrophy, as they are often amenable to transformation and gene disruption. The sequenced hemibiotrophs *M. grisea* (now named *M. oryzae*) (Jeon *et al.*, 2007), *U. maydis* (Kamper *et al.*, 2006) and *P. sojae* (Tyler *et al.*, 2006) or *P. capsici* are attractive models for ascomycetes, basidiomycetes and oomycetes, respectively. By studying these powerful systems, we will learn about the innovations that pathogens have evolved to interfere with host cell processes. This will not only benefit the field of plant–microbe interactions but should also enhance our general knowledge of plant physiology and cell biology.

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Chapter 5

GENOME BIOLOGY CRACKS ENIGMAS OF OOMYCETE PLANT PATHOGENS

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Abstract: All plant species, wild and cultivated alike, suffer from diseases. By far the most devastating plant pathogens are fungi and their look-alikes, the oomycetes. Oomycetes cause severe problems not only in agriculture but also in natural ecosystems. Best known is *Phytophthora infestans*, the species that caused the Irish potato famine in the mid-nineteenth century. Oomycetes and oomycete diseases have been the subject of numerous investigations, but the tactics exploited by these successful plant pathogens are still largely an enigma. In recent years, oomycete genomics uncovered a treasure trove of new information and that has enormously stimulated oomycete research. A major discovery was the highly diverse superfamily of secreted RxLR-dEER effectors that play important roles during plant infection. RxLR-dEER is a conserved motif in proteins encoded by oomycete avirulence genes that interact in a gene-for-gene manner with resistance genes. It has similarities to a motif in proteins secreted by malaria pathogens and helps targeting effectors into host cells. In this chapter some of the latest discoveries and insights into oomycete biology and pathology are presented. We describe several oomycete diseases and highlight species that feature as model organisms. We also summarize the genomic resources that are currently available and emphasize the impact of genomics on gene discovery in oomycetes. Finally, we refer to proteins secreted by oomycete pathogens and their potential roles in host–pathogen interactions.

Keywords: *Phytophthora*; effectors; oomycetes; genomics; avirulence gene; RxLR-dEER; secretome

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5.1 Introduction

Worldwide plants are attacked by a wide range of pathogenic oomycetes. *Phytophthora infestans*, the pathogen that was responsible for the Irish potato famine in the mid-nineteenth century, is one of many oomycete species that causes severe problems, not only in agriculture but also in natural environments and on indigenous trees and shrubs. In 1861, de Bary established for the first time that a filamentous organism that he named *P. infestans* was the cause of a plant disease (de Bary, 1876). Ever since, *Phytophthora* and other oomycetes have been the subject of numerous investigations but in spite of intense study, the tools and tactics exploited by these successful plant pathogens are still enigmatic. In recent years, genomics boosted oomycete research and genomic data uncovered a treasure trove for plant pathologists, genome biologists and evolutionary biologists alike. A major breakthrough was the discovery of the RxLR-dEER class of effector proteins. These effectors are highly diverse and are thought to play important roles during plant infection. Here we present some of the latest discoveries and insights into oomycete biology and pathology. The first part gives a brief overview of oomycete pathogens of plants and highlights the species that currently feature as model organisms. We then describe the genomic resources available for oomycete research and how genomics has accelerated gene discovery. The last part is devoted to various types of secreted proteins and the potential roles these proteins play in host–pathogen interactions.

5.2 Biology and pathology of oomycetes

5.2.1 Branches in the tree of life

Oomycetes, also known as water molds, resemble fungi in many ways. Like fungi, oomycetes have a global distribution and prosper in quite diverse environments. They can live as (hemi-) biotrophic or necrotrophic pathogens in association with plants (see Section 5.2.3; Box 5.1), animals or other microbes, but also as saprophytes feeding on decaying matter. Currently, at least 800 oomycete species are known, but depending on the definition of a species this number might actually reach 1500 (Dick, 2001). Nevertheless, the species richness seems low when compared to the number of fungal species known to date: 30 000 basidiomycete species have been described and ascomycetes reach a similar number (Kirk *et al.*, 2001; James *et al.*, 2006). It is, however, likely that there are many oomycetes out there yet to be discovered. In this respect, the genus *Phytophthora* is illustrative. In the last 10 years, at least 18 new species have been described, expanding the genus to nearly 95 members (Blair *et al.*, 2008; http://www.PhytophthoraDB.org, accessed June 2008).

Box 5.1 Lifestyles of plant pathogenic oomycetes

Plant pathogens can be divided into groups based on the different strategies they employ to colonize plants (Agrios, 2004). So-called obligate biotrophs grow and reproduce in living plant tissue and obtain energy by uptake of plant nutrients. They are entirely dependent on living cells and can therefore survive only by establishing a nondestructive relationship with their host. Among the obligate biotrophic oomycetes are the downy mildews (e.g. *Hyaloperonospora parasitica* and *Bremia lactucae*) and white rusts (e.g. *Albugo candida*). Necrotrophic pathogens feed on dead cell material. Before colonizing plant tissue, necrotrophs kill cells by secreting toxins or cell wall-degrading enzymes. A third strategy is an intermediate lifestyle called hemibiotrophy. The first stage of hemibiotrophy is based on an intimate relationship of the pathogen with its host. Later in the infection process, hemibiotrophic pathogens switch from their initial biotrophic to a destructive necrotrophic lifestyle. The oomycete genera *Pythium* and *Phytophthora* comprise both necrotrophs and hemibiotrophs.

Due to their shared morphology (filamentous, branched somatic structures that bear spores), oomycetes and fungi were traditionally classified in the same kingdom, the Fungi (Erwin and Ribeiro, 1996). In the 'Five Kingdom' classification of Margulis and Schwartz (2000), the two groups were separated into two kingdoms: Protoctista and Fungi, the first being a highly diverse group including both unikonts and bikonts. In recent years, new insights based on molecular phylogeny and comparative genomics have again reshaped the tree of life. In 2005, Keeling *et al.* launched the five supergroups of eukaryotes (Keeling *et al.*, 2005). In this classification, the ascomycete and basidiomycete fungi are grouped together with animals and Amoebozoa (e.g. slime molds) in the supergroup Unikonts (Fig. 5.1). Oomycetes with their biflagellated zoospores are typical bikonts. They fall within the supergroup Chromalveolates and the lineage Stramenopiles (Cavalier-Smith, 1999). Since the stramenopile lineage also contains photosynthetic organisms like diatoms and brown algae, it is thought that the common ancestor is a photosynthetic life form that obtained its chloroplast by secondary endosymbiosis, presumably from red algae in the bikont supergroup Plantae (Cavalier-Smith, 2002) (Fig. 5.1). The loss of plastids in the nonphotosynthetic stramenopile taxa is supported by whole genome comparisons that revealed numerous photosynthesis-related genes shared by *Phytophthora* and diatoms (Tyler *et al.*, 2006). Most of these genes encode mitochondria-targeted proteins with close homology to proteins targeted to chloroplasts in photosynthetic organisms.

Endosymbiosis can explain how genes with high similarity to bacterial genes end up in eukaryotes. In contrast, the processes underlying horizontal gene transfer (HGT) among eukaryotes are not so easy to trace. Nevertheless, as more and more genomes are sequenced, evidence for intraeukaryotic HGT is accumulating. In a thorough study, Richards *et al.* (2006) found strong evidence for HGT from ascomycete fungi to oomycetes. The genes involved

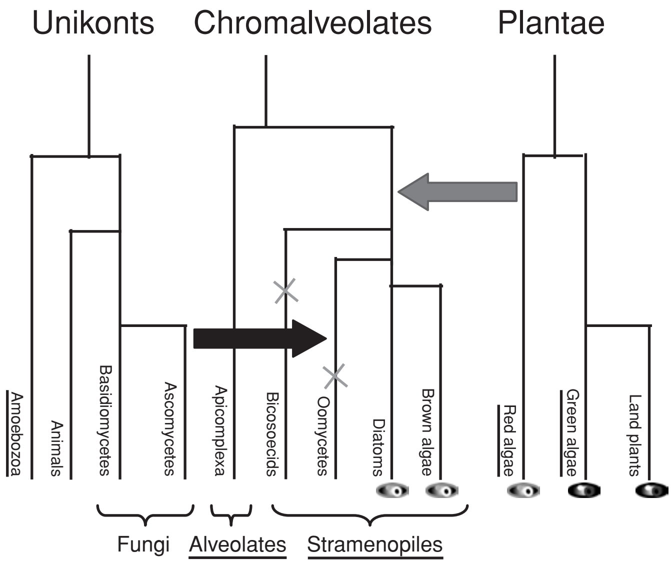

Figure 5.1 Plastid gain and loss and intra-eukaryotic horizontal gene transfer. This simplified phylogenetic tree shows three of the five eukaryotic supergroups as defined by Keeling *et al*. (2005). Oomycetes and Apicomplexa (among others *Plasmodium)* belong to the supergroup Chromalveolates. The supergroup Unikonts comprises, among others, animals and fungi, and the supergroup Plantae includes red and green algae as well as land plants. Two of the four bikont supergroups, Rhizaria and Excavates, are not shown. For simplicity, not all lineages are depicted. Major branches are underlined. During evolution the Chromalveolates acquired a photosynthetic plastid ( ) most likely originating from a red alga (gray arrow). This plastid was lost in the oomycete lineage (**x**) but retained in other chromealveolates that are phototrophic, for example the brown alga *Laminaria digitata* (kelp). *Phytophthora* genomes still carry many genes reminiscent of a phototrophic origin (Tyler *et al*., 2006). The black arrow represents HGT from ascomycetes to oomycetes. Genes found in oomycetes that are thought to have a fungal origin have putative functions in osmotrophy (Richards *et al*., 2006).

presumably have functions related to an osmotrophic lifestyle and could explain convergent evolution of osmotrophy coupled to filamentous growth in two distinct eukaryotic lineages. Convergent evolution probably also shaped the pathogenic behavior of oomycetes and fungi but whether, and to what extent, this involves HGT is still questionable (Latijnhouwers *et al*., 2003).

5.2.2 Typical features of oomycetes

Molecular phylogeny has now firmly established the distinct taxonomic positions of fungi and oomycetes, but also before the genomics era mycologists recognized several features that are characteristic for oomycetes (Erwin and Ribeiro, 1996). Among these are cell walls that lack chitin but are composed of a mix of cellulosic compounds and glycans, hyphae that lack septa (so-called

coenocytic mycelium) and have diploid nuclei (instead of haploid as found in fungi), stacked Golgi cisternae (versus unstacked in fungi), tubular mitochondrial cisternae (versus disclike in fungi) and sterol auxotrophy. Most characteristic for oomycetes are the zoospores, the free-swimming asexual spores that are propelled by two unequal flagellae and explain why a moist environment is most favorable for these water molds. One of the flagella has lateral hairlike structures called mastigonemes that contain the β-1,3-glucan mycolaminarin, an energy storage molecule that is also found in brown algae and diatoms (Feofilova, 2001). Literally, oomycetes means 'egg fungi', a name based on the egg-shaped resting spores, named oospores. Oomycetes can be either homothallic or heterothallic. Sexual reproduction is initiated upon release of hormones that trigger the formation of gametangia (♀ oogonium and ♂ antheridium) in which meiosis takes place. The diploid oospores are produced as a result of oogamous fertilization when a haploid oosphere fuses to a haploid gamete. Thick-walled oospores are most durable propagules that can survive harsh environmental conditions and are important for the generation and maintenance of genetic variation in a population. For a recent review on sexual reproduction in oomycetes, see Judelson (2007).

5.2.3 Oomycete diseases and model systems

The plant pathogenic oomycetes are remarkably diverse and exhibit lifestyles ranging from obligate biotrophic to necrotrophic (Box 5.1; Table 5.1). Oomycete diseases occur on nearly every agricultural crop across the globe and many of the economically important species were spread unwittingly by humans. The late blight pathogen *P. infestans*, renowned for its impact on history, caused the dreadful Irish potato famine in the 1840s. The journey from its origin to Europe and the subsequent global distribution was undoubtedly supported by potato trading. For a long time, Mexico was considered as the center of origin of *P. infestans* but recent findings raise doubts and point to South America (Grünwald and Flier, 2005; Gómez-Alpizar *et al.*, 2007). In the late 1870s, another severe oomycete disease appeared in Europe when lice-resistant (*Phylloxera*) grapevine rootstocks were imported from the US to France. Unfortunately, the rootstocks carried with them the downy mildew pathogen *Plasmopara viticola*. The disease was first observed in France in 1878 and in successive years it was found in almost every vineyard in Europe (Gobbin *et al.*, 2006). Another oomycete first described in the nineteenth century is *Bremia lactucae*, the causal agent of lettuce downy mildew (Regel, 1843). *B. lactucae* probably originates from Eastern Europe and the Near East, where the majority of the wild lettuce species is found. Nowadays, lettuce downy mildew occurs worldwide and is one of the major problems in lettuce cultivation. During the history of lettuce breeding, several resistance genes have been introgressed into commercial cultivars. However, the resistance did not last, probably due to rapid genetic adaptation of the pathogen

(Lebeda and Zinkernagel, 2003). Defeat of resistance by oomycete pathogens is not uncommon. Also, *P. infestans* is notorious in that respect (Wastie, 1991).

Unintended movement of oomycete plant pathogens is also the cause of severe diseases that disturb complete ecosystems. One illustrative example is sudden oak death caused by *Phytophthora ramorum*. This species originates from the Chinese highlands, and most likely made its entry into Europe, Northern America and Australia via trade of ornamental plants (Werres *et al.*, 2001; Goheen *et al.*, 2006). In California, sudden oak death emerged about a decade ago and killed many of the tanoaks (*Lithocarpus* spp.) and coast live oaks (*Quercus agrifolia*) that dominate in coastal forests. This disease not only harms its host plants but also indirectly changes avian species diversity (Monahan and Koenig, 2006). Another example is dieback in native Australian woods caused by *Phytophthora cinnamomi.* This species has an extremely wide host range (over 3000 plant species) and is believed to have originated near Papua New Guinea but now has a worldwide distribution. It was accidentally introduced into Australia around the 1920s. Dieback is threatening multiple endangered 'red list' plant species (http://www.iucnredlist.org) and also has a severe impact on some agricultural crops (Hardham, 2005; Shearer *et al.*, 2007).

Since *Phytophthora* diseases are so important and widespread, it is not surprising that they are intensively studied from different angles, the front runners being *P. infestans* (potato blight) and *Phytophthora sojae* (soybean root and stem rot). Also, downy mildews are favorite subjects of study, in particular in the field of molecular plant–microbe interactions. However, their obligate nature makes these organisms more difficult to handle. The many destructive *Pythium* species (>125) are mostly soilborne pathogens and primarily necrotrophs, and hence less attractive as a model for studying the intimate relation between host and pathogen. Table 5.1 gives an overview of a variety of oomycete pathogens. To choose a pathosystem that fulfills all requirements for a model system for research on molecular plant–microbe interactions is nearly impossible. In a recent review, Lamour *et al.* (2007) compared the five species for which the genomes have been sequenced – *P. sojae*, *P. ramorum*, *P. infestans*, *P. capsici* and *Hyaloperonospora parasitica* – and listed the pros and cons of these five species as model organisms for oomycete research. Attractive features include the high potential of *P. capsici* for genetic studies, the capability of *H. parasitica* to infect the model plant *Arabidopsis* and gene inactivation in *P. infestans* by gene silencing. In their comparison, Lamour *et al.* (2007) did not consider *Phytophthora brassicae.* It is true that its genome has yet to be sequenced but its potential as model species lies in the fact that, similar to *H. parasitica,* it is a pathogen of *Arabidopsis* and that different strains show differential interactions with various ecotypes (Roetschi *et al.*, 2001). The advantage over *H. parasitica* is its nonobligate nature and amenability to DNA transformation (Si-Ammour *et al.*, 2003). In that respect, the nonobligate *Aphanomyces euteiches* also has the potential to become a model species because one of its hosts is *Medicago*

Table 5.1 Oomycete diseases and model systems

Species	**Host plants**	**Disease type**	**Lifestyle**	**Model species**
Phytophthora infestans	Potato and tomato	Late blight	Hemibiotrophic	Yes
Phytophthora sojae	Soybean	Damping-off and root rot	Hemibiotrophic	Yes
Phytophthora ramorum	Several trees and bushes (e.g. oak, rhododendron)	Sudden oak death, canopy dieback	Hemibiotrophic	
Phytophthora nicotianae syn. *Phytophthora parasitica*	Multiple hosts, including citrus, castor bean and tobacco	Leaf and stem blight, root rot	Hemibiotrophic	
Phytophthora brassicae	Several brassicaceous plants, including *Arabidopsis thaliana*	Leaf blight	Hemibiotrophic	Potential
Phytophthora capsici	Multiple hosts, including cucurbits and capsicum peppers	Leaf blight, fruit, stem and root rot	Hemibiotrophic	Potential
Phytophthora palmivora	Large host range, including cacao and rubber tree	Leaf blight, fruit and root rot	Hemibiotrophic	
Phytophthora cinnamomi	Extremely large host range, exceeding 3000 species, including several crops	Root rot, dieback	Necrotrophic/ saprophytic	
Albugo candida	Several brassicaceous plants, like mustard and *Arabidopsis thaliana*	White rust	Obligate biotrophic	
Bremia lactucae	Lettuce and several closely related species	Downy mildew	Obligate biotrophic	
Hyaloperonospora parasitica	Several brassicaceous plants, including *Arabidopsis thaliana*	Downy mildew	Obligate biotrophic	Yes
Plasmopara viticola	Grapevine	Downy mildew	Obligate biotrophic	
Plasmopara halstedii	Asteraceae, including sunflower	Downy mildew	Obligate biotrophic	
Peronospora destructor	Monocots of the *Allium* family, like onion and garlic	Downy mildew	Obligate biotrophic	

Table 5.1 (*Continued*)

Species	Host plants	Disease type	Lifestyle	Model species
Pseudoperonospora cubensis	Several Curcubitaceae, like cucumbers	Downy mildew	Obligate biotrophic	
Sclerospora graminicola	Several monocots, maize, sorghum and pearl millet	Downy mildew	Obligate biotrophic	
Pythium ultimum	Multiple dicots (e.g. potato) and monocots (e.g. turf grass)	Damping-off	Necrotrophic	
Aphanomyces euteiches[a]	Several legumes, including peas, alfalfa, *Medicago truncatula* and clover	Root rot	Necrotrophic	Potential
Aphanomyces cochlioides[a]	Sugar beet	Root rot	Necrotrophic	

[a] Belongs to the order Saprolegniales.

truncatula, which is a model species for legumes and research on beneficial plant–microbe interactions (Gaulin *et al.*, 2007).

5.3 Genomic resources

The rise of genomics has had a major impact on oomycete research. In the early 1990s when the discipline of molecular phytopathology saw the light, research on oomycete pathogens lagged behind. The disadvantage of a diploid genome was one reason; homologous recombination, applicable in many fungi to make gene knockouts and mutant libraries for reverse genetics, is out of reach for an oomycete researcher. The large genome sizes of oomycetes and hence the inability to clearly separate chromosomes on agarose gels (Howlett, 1989; Tooley and Carras, 1992) as well as the lack of easy scorable phenotypic markers for genetic analyses were other barriers that made these organisms less attractive for basic research. Not without reason, Shaw (1983) referred to *Phytophthora* and related species as 'a nightmare for the fungal geneticist'. These disadvantages though were a stimulus to search for alternatives and the leap to genomics as an instrument to tackle *Phytophthora* was made in an early stage. As a result, the genomes of five oomycete species have now been sequenced and it is very likely that more will be sequenced in the next few years.

5.3.1 Expressed sequence tags and bacterial artificial chromosome libraries

Genomics activities started off with small-scale expressed sequence tag (EST) projects (Kamoun *et al.*, 1999; Qutob *et al.*, 2000) that were followed by the release of more extensive EST data sets (Randall *et al.*, 2005; Torto-Alalibo *et al.*, 2007). The *P. infestans* EST resource with over 94 000 ESTs from different isolates, developmental stages and culturing conditions is still one of the largest available for any plant pathogen (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html, accessedJune 2008). Table 5.2 summarizes the genomic resources for oomycete plant pathogens and lists the number of ESTs currently deposited in GenBank.

Bacterial artificial chromosome (BAC) libraries have been constructed for *P. infestans* (Randall and Judelson, 1999; Whisson *et al.*, 2001), *P. sojae* (Zhang *et al.*, 2006), *Phytophthora nicotianae* (Shan and Hardham, 2004) and *H. parasitica* (Rehmany *et al.*, 2003). Zhang *et al.* (2006) obtained the first genome-wide physical map of a *Phytophthora* species by integrating *P. sojae* BAC contigs with the genome sequence. BAC libraries have been instrumental for positional cloning, in particular of genes located at avirulence (*Avr*) loci (Allen *et al.*, 2004; Shan *et al.*, 2004; Armstrong *et al.*, 2005; Rehmany *et al.*, 2005; Jiang *et al.*, 2006c; van Poppel, *et al.*, in press). Also, the mating-type locus of *P. infestans* was mapped to a 60–70-kb region by exploiting BAC clones (Randall *et al.*, 2003). Several other cloning efforts were aided by the availability of BAC libraries (Laxalt *et al.*, 2002; Meijer *et al.*, 2005), and restriction and sequence analyses of individual BACs provided a first glimpse of the complexity and distribution of gene families (Liu *et al.*, 1998; Armstrong *et al.*, 2005; Jiang *et al.*, 2005) and repeat elements such as tRNA-related short interspersed elements, retrotransposons and DNA transposons (Ah Fong and Judelson, 2004; Jiang *et al.*, 2005; Whisson *et al.*, 2005).

5.3.2 Genome sequencing projects

To date, genome sequencing of five oomycetes has been (nearly) completed (Table 5.2). Four of these are *Phytophthora* species, number five is a downy mildew. Their genome sizes vary from 65 to 250 Mb and are overall larger than those of an average fungal genome. The first oomycete genome sequence project actually handled two species in parallel, *P. sojae* and *P. ramorum* (Govers and Gijzen, 2006; Tyler *et al.*, 2006), and at an early stage enabled the application of powerful analyses based on comparative genomics. This immediately revealed a high level of conserved synteny between the two species; but interestingly, the synteny appeared to be interrupted at loci harboring genes encoding effectors with putative functions in interactions with host plants (Jiang *et al.*, 2006a, 2008). Preliminary analyses including the three other sequenced genomes suggest that large regions of conserved synteny

Table 5.2 Genomic resources for plant pathogenic oomycetes

Species	Genome size (Mb)	Gene models	ESTs[a]	Genome sequencing and assembly status	URL[b]
Phytophthora infestans	237	22658	94091[c]	Completed	http://www.broad.mit.edu/annotation/genome/phytophthora_infestans
Phytophthora sojae	95	19027	28357[d]	Completed	http://genome.jgi-psf.org/Physo1_1/
Phytophthora ramorum	65	15743	–	Completed	http://genome.jgi-psf.org/Phyra1_1/
Phytophthora nicotianae/Phytophthora parasitica	n.d.	n.d.	553/6328[e]	–	–
Phytophthora brassicae	≈75[f]	n.d.	12922	–	–
Phytophthora capsici	65	n.d.	9	In progress	http://www.jgi.doe.gov
Hyaloperonospora parasitica	75	14726	46[g]	In progress	http://www.intlgenome.org/
Plasmopara halstedii	n.d.	n.d.	145	–	–
Pythium ultimum	n.d.	n.d.	9727	–	–
Aphanomyces euteiches	n.d.	n.d.	18684[h]	–	http://www.polebio.scsv.ups-tlse.fr/aphano/
Aphanomyces cochlioides	n.d.	n.d.	3599	–	–

n.d., not determined.
[a] http://www.ncbi.nlm.nih.gov/dbEST/.
[b] See also OMGN website, http://pmgn.vbi.vt.edu.
[c] Kamoun *et al*. (1999) and Randall *et al*. (2005).
[d] Qutob *et al*. (2000) and Torto-Alalibo *et al*. (2007).
[e] Panabières *et al*. (2005) and Le Berre *et al*. (2008).
[f] Roetschi *et al*. (2001).
[g] Casimiro *et al*. (2006).
[h] Gaulin *et al*. (2008) and Madoui *et al*. (2007).

occur throughout the genus *Phytophthora* and often even extend to *H. parasitica* (R.H.Y. Jiang and H.J.G. Meijer, personal communication). One example of a region of conserved synteny between *P. infestans* and *H. parasitica* that is interrupted by an effector gene (i.e. *Avr3a*) was published by Armstrong *et al.* (2005).

The phenomenon of conserved synteny also helps the gene-calling process. Over 20% of the predicted gene models in oomycetes are not known in other species, but the existence of orthologs often arranged in similar orders along the scaffolds is an indication that the gene models represent real genes. As shown in Table 5.2, the predicted number of gene models seems more or less collinear with genome size. Automated gene prediction is mostly based on training sets. The training set of oomycete genes is still relatively small and therefore the current genome browsers often show incorrect models. Handsaker *et al.* (2007) developed a gene-calling program called Orthosearch (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/GeneFinding.html) that makes use of the DNA conservation between *Phytophthora* species and could help to improve the gene prediction process. To avoid inaccurate models ending up in the literature and being propagated, it is important to check the coherence of existing data by comparing genome sequences of related species with ESTs and/or proteome data.

5.4 The impact of genomics on gene discovery

One incentive to sequence a genome is to accelerate gene discovery. Before the genomics era, the quest for the mechanisms that underlie pathogenicity in oomycetes already leads to the identification of a number of oomycete genes and gene families with putative roles in the interaction with host plants. For gene isolation in oomycetes, several strategies were used ranging from unbiased approaches, such as differential screening and positional cloning, to more biased approaches aimed at homologs of fungal pathogenicity genes or via reverse genetics starting with the purification of a protein that shows toxic or necrotic activity on a host plant. In this section, we give examples that demonstrate how genomics has influenced gene discovery in oomycetes.

5.4.1 From expression pattern to gene

The first *Phytophthora* genes to be identified were selected on the basis of expression patterns (Pieterse *et al.*, 1991, 1993a). These so-called *ipi* genes showed induced or specific expression during growth of the pathogen in association with its host (*in planta* induced) (Pieterse *et al.*, 1993b; van West *et al.*, 1998). Many years later, when comparison with the whole gene repertoire of *Phytophthora* became feasible, one of the *ipi* genes *ipiO* turned out to be member of the superfamily of RxLR-dEER effectors (see Section 5.5.3) and

to function as an avirulence (*Avr*) gene in a gene-for-gene manner with the resistance (*R*) genes *Rpi-blb1* and *Rpi-sto1* (Vleeshouwers *et al.*, 2008).

5.4.2 From protein to gene

Reverse genetics, successfully used to clone several *Avr* genes from fungal plant pathogens (Westerink *et al.*, 2004), was applied to clone the first elicitin gene named *para1* (Kamoun *et al.*, 1993). Elicitin was initially identified as a highly abundant toxic compound in culture filtrates of *Phytophthora parasitica* that causes necrosis on tobacco (Ricci *et al.*, 1989). The elicitin genes *para1* and *inf1*, from *P. parasitica* and *P. infestans*, respectively, are single copy genes that encode 10-kDa-secreted proteins with a core elicitin domain (Kamoun *et al.*, 1993, 1997). From a small-scale EST inventory, it became evident that the 10-kDa elicitins (ELIs) belong to a larger protein family comprising not only soluble extracellular ELIs but also ELIs anchored to the membrane or cell wall (Kamoun *et al.*, 1999). Subsequently, whole genome sequencing revealed that ELIs and elicitin-like (ELL) genes make up a large, complex and highly conserved family with 17 clades that existed prior to the divergence of *Phytophthora* species from a common ancestor (Jiang *et al.*, 2006b). Members of the 4 ELI clades all have elicitor activity on tobacco comparable to the ELI1 elicitins INF1 and PARA1, whereas ELL proteins are not active as elicitors (A. van 't Slot, P. van de Vondervoort and F. Govers, unpublished data). The intrinsic functions of ELI and ELL proteins are still unknown. However, ELIs can bind sterols and since *Phytophthora* is a sterol auxotroph, ELIs possibly function as carrier proteins to acquire sterols from the environment. The whole genome perspective tells us that the simplistic view that a single abundant component secreted in culture filtrate in an artificial environment (i.e. in vitro growth) is responsible for elicitin activity in the natural situation should be revisited.

A reverse genetics approach was also used to clone the gene encoding a 42-kDa extracellular glycoprotein from *Phytophthora megasperma* (Sacks *et al.*, 1995) that is now identified as a transglutaminase with the pathogen-associated molecular pattern (PAMP) pep-13 as the active site (Brunner *et al.*, 2002). Other examples are the elicitor CBEL (cellulose-binding elicitor lectin) from *P. parasitica* (Mateos *et al.*, 1997) in which two cellulose-binding domains act as PAMPs (Gaulin *et al.*, 2006) and NPP1 (necrosis-inducing *Phytophthora* protein 1) from *P. parasitica*, another putative PAMP (Fellbrich *et al.*, 2002) belonging to the class of Nep1-like proteins or NLPs (necrosis- and ethylene-inducing protein-like proteins). NLPs have a wide distribution across taxa and a broad spectrum of activity on plants (Gijzen and Nürnberger, 2006). In bacteria and fungi, NLPs are encoded by one or two genes but, in contrast, in *Phytophthora* species large families of NPP genes are found (Tyler *et al.*, 2006). As a last example, we mention the toxin PcF from *Phytophthora cactorum* (Orsomando *et al.*, 2001). Again, a single gene was cloned by reverse genetics but genomics data revealed a much more complex situation.

P. infestans homologs of PcF were named Scr74 (secreted cysteine-rich protein). The *scr74* family has at least ten members, some of which are clustered in the genome. They are highly polymorphic and under diversifying selection (Liu *et al.*, 1998). Also in *P. sojae*, *PcF* is a multicopy family but in *P. ramorum* very few *PcF* genes are detected (Tyler *et al.*, 2006).

5.4.3 From homolog to gene

The infection strategies of oomycetes and fungi have much in common (Latijnhouwers *et al.*, 2003) and therefore it is logical to investigate whether genes involved in host–pathogen interactions are also similar. The approach to isolate homologs of fungal pathogenicity genes by PCR amplification using degenerate primers was, for example, used by Götesson *et al.* (2002) to clone polygalacturonase genes from *P. cinnamomi*. Cell wall-degrading enzymes are important for the pathogenicity of many plant pathogenic fungi (ten Have *et al.*, 2002) and it is likely that necrotrophic oomycetes also make use of such enzymes. Laxalt *et al.* (2002) used degenerated PCR primers to clone the *P. infestans* gene coding for the Gα subunit of the heterotrimeric G-protein (i.e. *gpa1*). G-protein-mediated signal transduction is the most ubiquitous and best-studied signaling pathway among eukaryotes, and in several plant pathogenic fungi the Gα and Gβ G-protein subunits appear to be crucial for virulence. In *P. infestans*, silencing of *gpa1* results in nonpathogenic mutants (Latijnhouwers *et al.*, 2004) and silencing of *gpb1*, the gene encoding the G-protein β subunit, severely effects sporulation (Latijnhouwers and Govers, 2003). With the emergence of EST databases and genome sequences, it is no longer necessary to design the optimal degenerated PCR primers and to face the challenge of finding the perfect PCR conditions to clone the homologs. In the *Phytophthora* EST databases, several candidate genes that resemble known fungal pathogenicity genes have been identified and can be readily used for functional analysis (Qutob *et al.*, 2002; Randall *et al.*, 2005; Torto-Alalibo *et al.*, 2007).

5.4.4 From domain to gene

Mining the databases for certain motifs that represent catalytic domains of enzymes is another strategy that can lead to novel candidates. This was nicely demonstrated by Meijer and Govers (2006), who made an inventory of genes involved in phospholipid signaling in *Phytophthora*. They found several novelties including a family of genes encoding secreted proteins with a phospholipase D (PLD) catalytic domain. Similar proteins are present in downy mildews (H.J.G. Meijer, personal communication) but have, as yet, not been found in other plant pathogens or other eukaryotes. It is therefore interesting to investigate how widespread these enzymes are in oomycetes and whether they have a function in pathogenicity. Another novel class of proteins that deserves attention is the G-protein-coupled receptor phosphatidylinositol

(phosphate) kinases (GPCR-PIPKs) which are composed of an N-terminal seven transmembrane domain that is typical for GPCRs and a C-terminal PIPK catalytic domain. *Phytophthora* spp. and *H. parasitica* each have 12 GPCR-PIPKs which are slightly different. However, outside oomycetes only one homolog has been identified so far (Bakthavatsalam *et al.*, 2006). This is RpkA in *Dictyostelium discoideum* that is essential for cell density sensing (Bakthavatsalam *et al.*, 2007). Such a feature might also be important for oomycete pathogens when, for example, zoospores aggregate and prepare to invade the host. Involvement of G-protein signaling in this process was already demonstrated by Latijnhouwers *et al.* (2004), who found that zoospores of *P. infestans* transformants lacking the G-protein α subunit (generated through silencing of *gpa1*) have lost the ability to autoaggregate.

5.4.5 From map position to gene

For organisms that are a 'geneticist's nightmare' (Shaw, 1983), gene discovery based on positional cloning is not the most logical approach to follow. Nevertheless, before the genomics era, several groups started to generate molecular genetic linkage maps with the aim to clone genes with a scorable phenotype but no clue about the gene product or stage of expression. Often their genes of interest were *Avr* genes that interact in a gene-for-gene manner with plant *R* genes. In addition, the mating-type locus is a target for positional cloning (Randall *et al.*, 2003). Species for which molecular genetic linkage maps have been generated are *P. infestans* (van der Lee *et al.*, 1997; Carter *et al.*, 1999; van der Lee *et al.*, 2001; Randall *et al.*, 2003; van der Lee *et al.*, 2004), *P. sojae* (Whisson *et al.*, 1995; MacGregor *et al.*, 2002; May *et al.*, 2002; Whisson *et al.*, 2004), *H. parasitica* (Rehmany *et al.*, 2003) and *B. lactucae* (Sicard *et al.*, 2003). These long-term investments paid off, and at least a handful of *Avr* genes have been isolated on the basis of their map positions. One example where cloning primarily relied on RAPD (random amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism) markers, a large number of recombinants and chromosome walking, was *Avr1b*-1 from *P. sojae* (Shan *et al.*, 2004). *P. sojae* is homothallic and once F1 hybrids between two different races have been identified, it is straightforward to obtain F2 mapping populations by selfing the F1 hybrids (Tyler *et al.*, 1995; May *et al.*, 2002). In most cases, however, the genetically linked markers were not sufficient to locate the gene – the distance was too large or too few recombinants were available. Additional markers obtained by transcriptional profiling, such as cDNA-AFLP (Guo *et al.*, 2006) or by suppression subtractive hybridization (Bittner-Eddy *et al.*, 2003), were needed to nail down the gene of interest and in several cases genomics resources helped to speed up the gene isolation. In the case of *P. infestans Avr4* ESTs matching, short cDNA-AFLP fragments were instrumental (van Poppel *et al.*, in press). To find a candidate gene at the *P. infestans Avr3b-Avr10-Avr11* locus, Jiang *et al.* (2006c) made use of a *Phytophthora* GeneChip with over 18 000 unigenes. The chip was used

for transcriptional profiling and comparative genomic hybridization (array-CGH), and this provided additional markers that were crucial for identifying the *Pi3.4* gene.

5.4.6 From effector activity to gene

Nearly all the *Avr* genes obtained by positional cloning encode proteins that belong to the superfamily of RxLR-dEER effectors (see Section 5.5.3; Chapter 4). This finding stimulated the use of a gene discovery approach based on *in planta* expression of effector genes. The principle of this approach was first described by Torto *et al*. (2003) who mined *P. infestans* ESTs for sequences encoding secreted proteins and cloned these in binary potato virus X (PVX) vectors for *in planta* expression in *Nicotiana benthamiana*. This high-throughput approach resulted in the identification of *crn1* and *crn2* (crinkling and necrosis). When expressed *in planta*, *crn1* and *crn2* cause necrosis and *crn2* also induces expression of defense response genes in tomato. The ESTs that were tested by Torto *et al*. (2003) encode a variety of secreted proteins including cell wall-degrading enzymes, proteases, a chitinase and elicitins (ELI and ELL proteins). With the prediction that a putative *Avr* gene is likely a member of the RxLR-dEER family, Vleeshouwers *et al*. (2008) developed a more dedicated approach concentrating exclusively on RxLR-dEER effectors. The corresponding ESTs were extracted from the *P. infestans* EST database and cloned into a binary PVX vector. In a high-throughput screening, a broad range of wild *Solanum* accessions showing resistance to late blight was toothpick-inoculated with the *Agrobacterium* strains carrying recombinant PVX constructs in a binary plasmid. Effectors inducing necrosis were retested on F1 progeny obtained by crossing a responsive, resistant accession with a nonresponsive, susceptible potato line. If the progeny showed cosegregation of resistance and response to the effector, the resistant accession was further investigated for the presence of an *R* gene that specifically recognizes the RxLR-dEER effector. This approach, dubbed as 'effector genomics', resulted in the identification of an *R* gene in *Solanum stoloniferum* that is responsible for recognition of IPI-O (in planta-induced O). The *R* gene *Rpi-sto1* turned out to be the ortholog of *Rpi-blb1*, and hence *ipiO* is now known to function as *Avr-blb1* and *Avr-sto1* (Vleeshouwers *et al*., 2008).

Effector genomics is clearly a powerful approach that will soon result in the identification of many more effectors matching the wide range of *R* genes present in the *Solanum* gene pool or in any gene pool that is exploited for crop breeding. It is, however, a very biased approach; only genes that have certain characteristics such as signal peptide sequences and particular domains known to be involved in effector function are taken into account. Despite the fact that positional cloning is tedious, it should not be discarded in the gene discovery process. The *pi3.4* gene located at the *Avr3b-Avr10-Avr11* locus in *P. infestans* is an illustrative example of a gene that would have been missed by effector genomics (Qutob *et al*., 2006; Jiang *et al*., 2006c). *Pi3.4*

encodes a protein of 1956-amino acids with regulatory domains characteristic for transcription factors. In isolates avirulent on *R3b*, *R10* and *R11* potato plants, truncated versions of *Pi3.4* are amplified with up to 25 copies located in tandem and adjacent to the full-length copy of *Pi3.4*. Sequence comparison revealed that the amplification may generate modular diversity and assist in the assembly of novel full-length genes via unequal crossing over. Such amplification is not found in virulent isolates. The current hypothesis is that *Pi3.4* is a transcriptional regulator that influences expression of effector genes, for example RxLR-dEER genes. Copy number variation and modular diversity as observed at the *Avr3b-Avr10-Avr11* locus could be a mechanism for pathogens to quickly adapt to the environment.

5.5 The secretome

Plant pathogens secrete numerous effector molecules that fulfill important roles in mediating pathogenesis and scouting for targets in the host to deploy their action. The secretome is defined as the sum of all proteins secreted by an organism. Effectors function at the forefront to establish adhesion, penetration of host tissue and degradation of cell walls. Moreover, effectors interact with plant cell components at the cell wall, plasma membrane or in the cytoplasm, thereby reprogramming the host cell to accommodate the needs of the pathogen. For biotrophic pathogens, the fine-tuning of this reprogramming is very important. They probably have to make compromises: the host cell has to stay alive and should support the pathogen in its urge to survive. Hence, the pathogen has to be able to suppress basal defense responses (PAMP-triggered immunity or PTI) triggered by effectors known as PAMPs (Jones and Dangl, 2006). Once these barriers are overcome, the pathogen may face another barrier, that is, the effector-triggered immunity. Effector-triggered immunity, however, is triggered only when the plant possesses *R* genes that monitor specific effectors. Figure 5.2 gives a simplified view of the interplay between plant and pathogen in a compatible and incompatible interaction.

Genome-mining expeditions focused on the secretome of *Phytophthora* resulted in numbers ranging from 1188 secreted proteins in *P. ramorum* to 1975 in *P. infestans*, with *P. sojae* (1464) as intermediate (Jiang, 2006; Tyler *et al.*, 2006; R.H.Y. Jiang, personal communication). The secretome of the diatom *Thalassiosira pseudonana*, the closest relative of oomycetes that is sequenced and not pathogenic, is much smaller and lacks many of the enzymes found in *Phytophthora* (Tyler *et al.*, 2006). More than 80% of the genes encoding secreted proteins (defined as *spe* genes) belong to gene families and they often occur in clusters. The percentage of *spe* orthologs is lower than that among non-*spe* genes, indicating that the secretome evolved at a faster pace than the average genome. This supports the idea that many of the *spe* genes are involved in molecular 'arms races'. They undergo rapid changes that are driven by

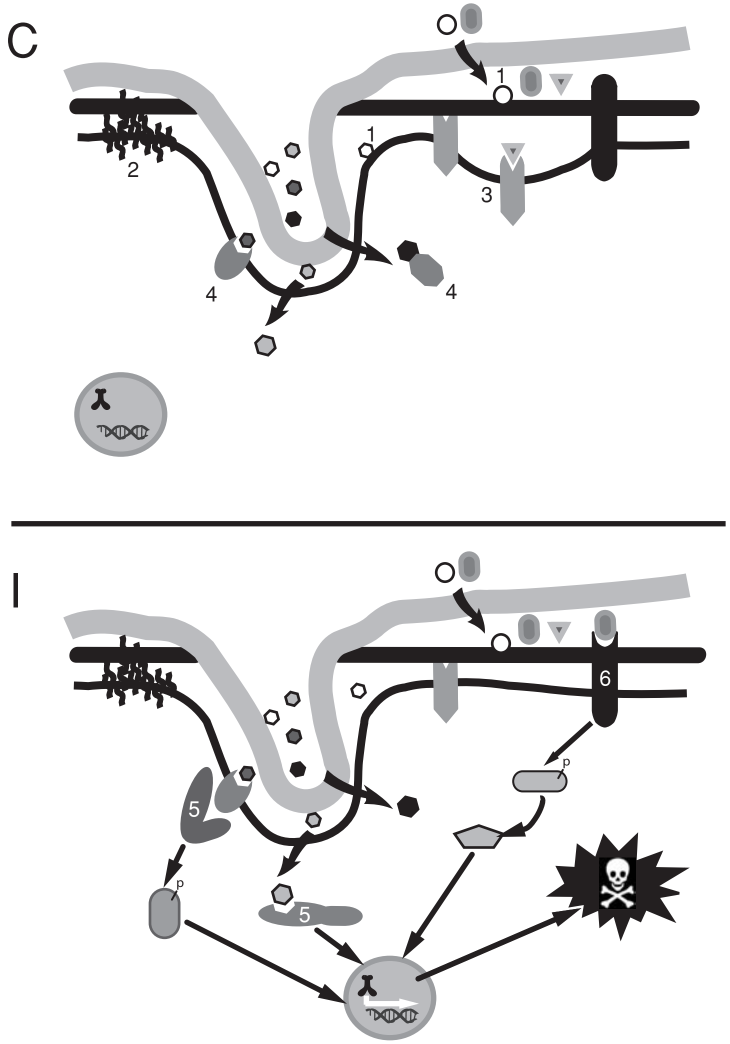

Figure 5.2 During a host–pathogen interaction, effectors (⬡⬡▯▽) are secreted by the pathogen and directed toward their targets. These targets can be either membrane-spanning or cytoplasmic proteins or structural molecules such as cell wall components. Effectors are needed to establish a compatible interaction (C) and are active in different steps of the infection process, for example attachment (1), breakdown of the cell wall by enzymes (2), relaxation of the cell membrane (3) or alteration of host metabolism (4). During an incompatible interaction (I), the actions of effectors are monitored by a warning system in the host. When an effector is detected by an R protein (5) or by a receptor-like kinase (6), signaling cascades are initiated, leading to transcriptional changes and activation of defense responses, and in many cases resulting in cell death. The figures are simplified, showing only the cross talks discussed in this chapter. The system is too complex to show all steps known to be involved in host–pathogen interactions.

diversifying selection. Within the secretome, however, individual families appear to have evolved at different rates. NPPs and ELIs are highly conserved and this could be due to selection pressure exerted on certain domains, for example to maintain enzymatic functions. In contrast, the RxLR-dEER proteins as well as some families containing proteins composed of repeats are highly divergent (Jiang, 2006).

In a recent review, Kamoun (2006) presented an extensive catalog of oomycete effectors. In the next sections, we touch upon a few classes of secreted proteins that were not included in that review. We also elaborate on the class of RxLR-dEER effectors whose discovery in 2004 has created a flurry of new activity in the research community.

5.5.1 Adhesins

When zoospores attach to the plant surface, they adopt a specific orientation before they encyst. The ventral surface faces the plant. During and after encystment, the spore secretes adhesive material via exocytosis of peripheral vesicles (Hardham, 2007). The ventral vesicle contains a high-molecular-weight adhesive protein named Vsv1 that attaches the cyst to the plant. Cloning of the *Vsv1* gene from *P. cinnamomi* revealed that the protein PcVsv1 carries 47 thrombospondin type 1 repeats (Robold and Hardham, 2005). Thrombospondin type 1 repeat is present in a variety of adhesive molecules secreted by mammalian cells and apicomplexan parasites such as malaria, but is not found in any fungal or plant protein. *PcVsv1* is a single copy gene. Other *Phytophthora* species have a PcVsv1 homolog, but in the *H. parasitica* genome sequence no homolog could be detected. The closest homolog outside the genus was found in the apicomplexan parasite *Cryptosporidium parvum*, suggesting that apicomplexans and oomycetes have similar types of adhesins.

Other types of adhesins that were identified in *Phytophthora* are mucins. Also, these adhesins are composed of variable numbers of repeats. The sizes of the repeat differ per mucin: some have repeats of 45 amino acids, others of only 10 amino acids. Görnhardt *et al.* (2000) cloned two *P. infestans* mucin genes that showed specific expression in germinated cysts. Meijer *et al.* (2006), who used a proteomics approach, identified six different mucins (and mucin-like proteins) associated with the *P. ramorum* cell wall. The gene models showed that some of these are encoded by multigene families.

CBEL is another protein that is involved in the attachment of *Phytophthora* to the host surface (Mateos *et al.*, 1997). It was first identified in *P. parasitica* var. *nicotianae.* The two cellulose-binding domains not only act as PAMPs but also bind to cellulose and tobacco cell walls (Gaulin *et al.*, 2006). Silencing of the *CBEL* gene did not impair mycelial growth or virulence but the transformants showed a reduced attachment to cellulose (Gaulin *et al.*, 2002).

5.5.2 Phospholipases

Phospholipases are a diverse group of enzymes that break down specific bonds in phospholipids (see Chapter 10). Phospholipase C (PLC) and D (PLD) also have functions in signal transduction and are both involved in the production of phosphatidic acid (PA), an important second messenger in many organisms. Biochemical studies by Latijnhouwers *et al*. (2002) showed that PLD and PA play a role in zoospore encystment in *Phytophthora*, but no PLC activity was found. The *Phytophthora* genome sequences revealed 18 gene models for PLD, but surprisingly no model for PLC (Meijer and Govers, 2006). This is unique since all other eukaryotes sequenced to date have one or more *PLC* genes. In contrast, the diversity in the types of PLDs is remarkably high and possibly one of these PLDs compensates for the lack of a PLC. Only 1 of the 18 PLD models matched to a known PLD of the class PXPH-PLD, whereas 2 others encode novel proteins in which the PLD catalytic domain is combined with transmembrane domains (PXTM-PLD and TM-PLD). Of the remaining 15, 14 encode a PLD with a slightly aberrant catalytic domain and 12 have an N-terminal signal peptide. The secreted ones, named sPLD-likes, belong to the secretome.

Phospholipases in the secretome are not exceptional. Phospholipases A and B are secreted by several organisms and also PLD is described as being secreted by bacteria. It has been postulated that PLDs in bacteria act as virulence factors (Songer, 1997) and the human pathogen *Neisseria gonorrhoeae* was reported to secrete a PLD that enhanced complement receptor-3-mediated invasion of cervical epithelial cells (Edwards and Apicella, 2006). However, PLD secreted by a plant pathogen has not been reported before and none of the sPLD-likes found in *Phytophthora* has homologs in fungi or other eukaryotes. Recent findings in our laboratory show that PLD activity is present in the extracellular fluid of *P. infestans* and that specific phospholipids in plant membrane vesicles are degraded in in vitro assays. A function for the sPLDs in degradation of plant tissue is therefore plausible (H.J.G. Meijer, personal communication).

5.5.3 RxLR-dEER effectors

When the first few oomycete *Avr* genes were isolated by positional cloning, the first two *Phytophthora* genome sequences were being released. A genome mining and alignment expedition during a genome annotation jamboree in 2004 at the Joint Genome Institute resulted in the discovery of a conserved motif in all Avr's and Avr homologs (Govers and Gijzen, 2006). This motif located adjacent to the signal peptide was christened as RxLR-dEER. Since no conserved motifs have been identified in fungal avirulence proteins, the presence of a conserved motif in oomycete Avr proteins came as a surprise. There is now ample evidence that the RxLR-dEER superfamily (collectively called the RxLR-dEER effectome) comprises most, if not all, effectors that interact

in a 'gene-for-gene' manner with *R* genes. Table 5.3 provides a summary of eight oomycete Avr factors, six of which were obtained by positional cloning. Avr3a was characterized by means of association genetics (Armstrong *et al.*, 2005) and IPI-O was identified as an Avr protein in an effector genomics screen (Vleeshouwers *et al.*, 2008). Several new Avrs are in the pipeline and can soon be added to this list.

The number of RxLR-dEER effectors found in each of the sequenced genomes is astonishing. Jiang *et al.* (2008) reported 385 different RxLR-dEER proteins in *P. sojae* and 370 in *P. ramorum*. With the same mining strategy of reiterated BLAST searches and hidden Markov models, around 562 members were found in *P. infestans* (R.H.Y. Jiang, personal communication). Lately, several publications have listed different numbers and this is likely due to the different algorithms that are used by the different groups (Lamour *et al.*, 2007; Whisson *et al.*, 2007; Win *et al.*, 2007; Jiang *et al.*, 2008). Dispute about the best mining strategy continues, but there is no doubt that the family is extremely large and diverse. The N-terminal part with the signal peptide and the RxLR-dEER motif is conserved, but in the C-terminal regions there is almost no similarity. Avr function and diversity are two characteristics that underscore the role of RxLR-dEER effectors in host specificity. Another characteristic is the distribution of RxLR-dEER genes over the genome. The high level of conserved synteny between two *Phytophthora* species over large segments of the genome is disrupted by indel blocks and nearly all RxLR-dEER genes are located in these blocks (Jiang *et al.*, 2006a, 2008). The genomes are highly dynamic and full of transposons, and it is remarkable that RxLR-dEER genes are more often flanked by transposon-like sequences than other genes (R.H.Y. Jiang and M.C. Zody, personal communication). Apparently, transposition is one of the factors that contributed to the rapid evolution of these large effector families.

At the time of discovery the function of the RxLR-dEER motif was a mystery, but shortly after the 2004 jamboree Hiller *et al.* (2004) and Marti *et al.* (2004) reported the existence of a conserved motif, named PEXEL/VTS, in secreted effectors of the alveolate parasite *Plasmodium falciparum*, the causal agent of malaria. RxLR-dEER resembles the PEXEL/VTS motif, in both sequence and location in the protein (Fig. 5.3a). The PEXEL/VTS motif was shown to be involved in host cell targeting of effectors. Via this system, the parasite delivers a secretome consisting of an estimated 400 proteins into the cytoplasm of erythrocytes, presumably to carry out virulence and host-remodeling functions (Hiller *et al.*, 2004; Marti *et al.*, 2004). This led to the hypothesis that RxLR-dEER effectors are also targeted into host cells. Bhattacharjee *et al.* (2006) strengthened this hypothesis by showing that the RxLR motif of *P. infestans* Avr3a, and not a mutated motif, can functionally complement a PEXEL/VTS motif in *Plasmodium*. More recently, Whisson *et al.* (2007) were able to demonstrate that *P. infestans* transformants carrying an *Avr3a* transgene with a mutated RxLR-dEER motif cannot restore the Avr phenotype of a race 3 strain, adding support to the idea that Avr3a has to

Table 5.3 An overview of known oomycete avirulence genes

Effector gene	Origin	*R* gene	Type of R protein	WYL domains	Protein variants	Positive selection	Types of mutations affecting avr phenotype
HpATR1^{NdWsB} [a]	*Hyaloperonospora parasitica*	*RPP1*	TIR-NB-LRR	—	8	Yes	SNPs, indels
HpATR13[b]	*Hyaloperonospora parasitica*	*RPP13-Nd*	CC-NB-LRR	—	6	Yes	SNPs, indels
PsAvr1a[c]	*Phytophthora sojae*	*Rps1a*	n.d.	W	n.d.		n.d.
PsAvr1b-1[d]	*Phytophthora sojae*	*Rps1b*	n.d.	WY	4	Yes	Transcript regulation, SNPs
PsAvr3a[e]	*Phytophthora sojae*	*Rps3a*	n.d.	—	n.d.		n.d.
PiIpiO[f]	*Phytophthora infestans*	*Rpi-blb1*, *Rpi-sto1*	CC-NB-LRR	W	10	Yes	SNPs
PiAvr3a[g]	*Phytophthora infestans*	*R3a*	CC-NB-LRR	W	2		SNPs
PiAvr4[h]	*Phytophthora infestans*	*R4*	n.d.	WWWY	6		SNPs, frameshifts

n.d., not determined.
[a] Rehmany *et al*. (2005).
[b] Allen *et al*. (2004).
[c] Qutob *et al*. (2007).
[d] Shan *et al*. (2004).
[e] Tedman-Jones *et al*. (2007).
[f] Vleeshouwers *et al*. (2008).
[g] Armstrong *et al*. (2005).
[h] van Poppel *et al*. (in press).

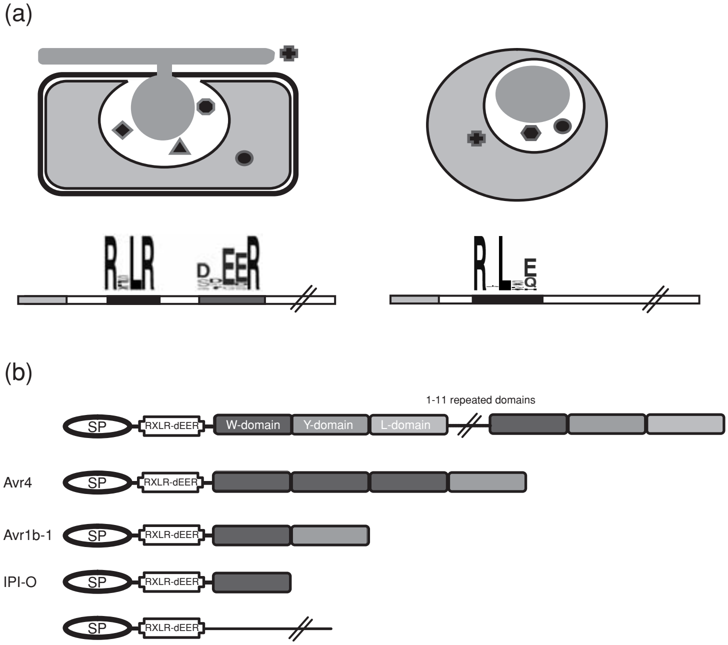

Figure 5.3 (a) Infection strategies of plant pathogenic oomycetes and the malaria parasite *Plasmodium* show several similarities. Biotrophic oomycetes penetrate plant cells by forming haustoria (left). *Plasmodium* is localized inside erythrocytes within parasitophorous vacuoles (PV) (right). Both the haustorium and the parasitophorous vacuoles are surrounded by a host-derived membrane. The pathogens secrete effectors into the apoplast or extrahaustorial space (oomycetes) or the PV (*Plasmodium*). Effectors equipped with a host cell-targeting motif can enter host cells. The host cell-targeting motifs, RxLR-dEER in oomycetes and PEXEL/VTS in *Plasmodium*, are located N-terminal and adjacent to the signal peptide (marked in light gray). The dEER motif is conserved in most, but not all, oomycete RxLR effectors. The sequence logos show the amino acid conservation in a random set of effectors. The C-terminus in these effectors is highly diverse in both sequence and length. (b) In many of the RxLR-dEER effectors the C-terminus carries a variable number of domains that may occur in a repeated fashion (top row) (Jiang *et al*., 2008). Three domains are defined and named W, Y and L after the most conserved amino acid in that particular domain. Different combinations of domains are found. In a subset of the effectors, the domains are lacking (last row). The rows in between show the domain composition of three RxLR-dEER effectors and illustrate the possible variations in the C-terminus. Avr4 and IPI-O from *Phytophthora infestans* and Avr1b-1 from *Phytophthora sojae* are recognized as Avr proteins in plants carrying the cognate *R* genes.

reach the cytoplasm to be recognized by the intracellular CC-NB-LRR protein R3a. They also transformed *P. infestans* with reporter constructs consisting of β-glucuronidase (GUS) fused to wild type and mutated forms of the RxLR-dEER motif and monitored the intracellular location of GUS after infection. GUS is not stable in the apoplast, and indeed only in the presence of the wild-type motif the infected cells stained blue. Similar results were obtained

by Dou *et al.* (2008a,b) with Avr1b-1 from *P. sojae.* These experiments strongly support the notion that the RxLR-dEER motif indeed can function as a host cell-targeting signal.

What then is the function of these effectors when they end up in the host cell and how do they modulate the host cell machinery? The high diversity of the C-terminus of RxLR-dEER proteins is consistent with the notion that this part is the effector domain that is active in the cell. For *P. infestans* Avr3a, Bos *et al.* (2006) showed that recognition by R3a is based on the last 75 amino acids of the avirulent variant of Avr3a, Avr3a^{KI}, and that the N-terminal region is dispensable for recognition. The C-terminal part also suppresses HR induced by elicitin INF1 in *N. benthamiana*, suggesting that the presence of Avr3a^{KI} confers a selective advantage to the pathogen when infecting a susceptible host. The virulent variant, Avr3a^{EM}, is not recognized by R3a and is, surprisingly, unable to suppress INF1-induced HR. As in Avr3a, the RxLR-dEER domains in Avr4 (van Poppel *et al.*, in press) and *P. sojae* Avr1b-1 (Dou *et al.*, 2008a,b) are also dispensable for Avr function.

Moreover, in several of the Avr proteins, the C-terminal domain is under diversifying selection (Table 5.3). As yet, however, there is no clue about the molecular or biochemical function of the RxLR-dEER effectors and in the C-termini of the investigated effectors, no catalytic domains or ProSite motifs can be distinguished. Interestingly, motif searches and hidden Markov model screening revealed three conserved domains (W, Y and L) that are repeated (Jiang *et al.*, 2008). W, the most prominent domain, consists of 25 amino acids and occurs in 60% of all RxLR-dEER members. Up to 11 repeats were found (Fig. 5.3b) and the number correlates with the length of RxLR-dEER proteins. The different numbers of repeats may, at least partly, explain the divergence between paralogs, and by tracing the evolutionary history of the RxLR-dEER genes it might be possible to find correlations with their specific activities as an Avr factor. In a subset of the RxLR-dEER effectors, no W, Y or L domains can be distinguished; an example is ATR13 of *H. parasitica* (Table 5.3). Instead, ATR13 has a heptad repeat region and a direct repeat region, but as with W, Y and L domains, the relevance of these repeats is not clear (Allen *et al.*, 2004). Also, in the RxLR-dEER domain, some peculiarities should be noted. Supposedly, this domain interacts with other proteins at the cell membrane to activate an uptake machinery or to induce the formation of channels. Those complexes might involve other pathogen proteins. Crinkling and necrosis-inducing (CRN) proteins have a highly conserved motif LXLFLAK and this motif was found to overlap with the RxLR motif of 13 RxLR proteins in *H. parasitica*. RxLR proteins with a CRN domain are absent in *Phytophthora*, suggesting that these proteins recently evolved (Win *et al.*, 2007). Furthermore, IPI-O has an RGD (arginine–glycine–aspartic acid) motif that overlaps with the RxLR motif. Interestingly, RGD in IPI-O has been shown to bind to an *Arabidopsis* lectin receptor kinase (LecRK79) (Gouget *et al.*, 2006) and this LecRK could well be an effector target that mediates the uptake of RxLR-dEER effectors into the host cell. LecRK79 plays a role in the

cell wall–plasma membrane adhesions and IPI-O was shown to disrupt these adhesions in *Arabidopsis* (Senchou *et al.*, 2004). Consistent with the idea that RxLR-dEER effectors have virulence functions, overexpression of *ipiO* in *Arabidopsis* Col-0 leads to gain of susceptibility for *P. brassicae* strains that cannot infect wild-type Col-0 plants (K. Bouwmeester and F. Govers, unpublished data).

5.6 Future perspectives

A decade ago molecular genetic research on oomycetes was still in its infancy. Only a few genes were described, there were hardly any resources and the molecular toolbox was limited. Genomics has dramatically changed our way of thinking and has already uncovered many secrets about biology, pathology and evolution of oomycetes. Yet there are still many enigmas that need to be cracked and therefore the various -omics approaches should be further exploited. Oomycete proteomics has already started and the data obtained from expressed peptide tags (Savidor *et al.*, 2006) are now being integrated to genome annotations. Peptides that do not fit existing gene models show that the annotation needs further optimization. Also, transcriptomics is going strong. Data obtained with the first-generation *P. infestans* DNA chip have already been used for various purposes (Jiang *et al.*, 2006c; Prakob and Judelson, 2007; Judelson *et al.*, 2008). With the large reservoir of rapidly evolving RxLR-dEER effectors, effectomics is also feasible. The diversity seen in strains that have been sequenced is just the tip of the iceberg. The number of variants of each RxLR-dEER effector in field isolates varies (Table 5.3). For some, just two allelic variants are found in a wide range of isolates. For others, already more than ten variants have been found in a limited set of isolates. High-throughput sequencing such as with the 454 or Solexa systems should be exploited to monitor the RxLR-dEER effectome of larger sets of isolates. Also resequencing of isolates that originate from different regions or host plants will provide valuable insights into the specialization of such isolates.

To date, oomycete genomics is still largely focused on *Phytophthora.* It will be exciting to unravel the genomes of a wider range of oomycete species, in particular species infecting animals and insects. This will offer many new scientific challenges and provide opportunities to tackle these notorious pathogens.

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Chapter 6

PLANT–VIRUS INTERACTIONS: DEFENCE AND COUNTER-DEFENCE

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Abstract: Viruses replicate in intimate association with their host cells. This has influenced the means by which plants defend themselves against viruses and the strategies adopted by viruses to exploit host plants. In this chapter, we examine how *R* gene-mediated resistance against specific avirulent viral pathogens differs in important respects from resistance against non-viral pathogens (bacteria, fungi, oomycetes, etc.) while utilising many of the same signal transduction pathways. We also explore mechanisms of recessive gene-mediated resistance to viruses and genetically determined resistance to viral movement. Finally, we describe the emergence of host RNA silencing pathways as key determinants of the outcome of most plant–virus interactions. These pathways can act as important effectors of basal and induced resistance to viruses. Additionally, RNA silencing pathways can act as targets for viral suppressor proteins. Disruption of silencing pathways is the predominant means by which viruses induce disease symptoms in susceptible plant hosts.

Keywords: salicylic acid; RNAi; hypersensitive response; micro-RNA; induced resistance; short-interfering RNA; alternative oxidase; RNA-directed RNA polymerase; jasmonic acid; argonaute; NBS-LRR; Dicer-like protein

6.1 The unique challenge posed by viruses to plants

Viruses are obligate intracellular parasites that replicate in intimate association with a host cell. They do not separate themselves from the host cell with impermeable or semipermeable membranes or walls, and they must exploit the translational machinery of the host cell to synthesise viral proteins (Hull, 2002). This inextricable relationship between host and virus confronts plants with challenges that are different in important respects to those

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presented by cellular pathogens, such as fungi, oomycetes and bacteria, that to a greater extent can live independently of the host. These considerations have almost certainly influenced the co-evolution of plants and the viruses that infect them in ways that are not seen in other plant–pathogen relationships. In this chapter, we discuss the current state of knowledge of resistance to plant viruses and how in some cases viruses have evolved mechanisms to overcome or subvert resistance.

6.2 Virus infection and propagation through susceptible host plants

6.2.1 Viral replication and gene expression

Most plant viruses have RNA genomes, of which the majority are positive sense single-stranded (ss) RNA. Replication of these viruses requires RNA-dependent RNA polymerase activity provided by a replicase complex formed by recruitment of virus- and host-encoded proteins, with the virus providing the catalytic moiety (Buck, 1996; O'Reilly and Kao, 1998). Hereafter, RNA-dependent RNA polymerase is abbreviated to RdRp in the context of viral replicase complexes. These should be distinguished from host-encoded RNA-dependent RNA polymerases, which are abbreviated to RDRs (Sections 6.3.3 and 6.4). Replication proceeds via synthesis of a negative strand complementary RNA to act as template for positive sense RNA and for some viruses as the template for subgenomic mRNA synthesis. For a few positive sense viruses, the initiation of RNA synthesis by the viral RdRp may require a short RNA primer molecule (Buck, 1996; O'Reilly and Kao, 1998; Kao *et al.*, 2001). Replicase complexes assemble on intracellular membranes, which become modified to provide an optimal environment for production of viral genomic RNA and mRNA. Membrane modification also minimises exposure to host surveillance and resistance mechanisms, particularly those based on RNA silencing (Schwartz *et al.*, 2004; Ahlquist, 2006) (Section 6.4).

Some plant viruses possess DNA genomes, of which the best studied are the geminiviruses and caulimoviruses (Hull, 2002). Geminiviruses have ss circular DNA genomes, replicate in the host cell nucleus using host DNA polymerase activity, and use host RNA polymerase II to generate their mRNAs (Stanley, 1985; Hull, 2002). A characteristic symptom of geminivirus infection is triggering of host cell division. Since most plant cells are fully differentiated and contain little or no DNA polymerase, resumption of the cell cycle results in increased DNA polymerase activity, which is required by the virus for replication of geminivirus DNA (Gutierrez, 2002; Hanley-Bowdoin *et al.*, 2004).

Caulimoviruses, for example cauliflower mosaic virus (CaMV), are pararetroviruses: double-stranded (ds) DNA viruses that replicate via a

single-stranded RNA molecule (Hull, 2002). Following infection of a host cell, the nicked dsDNA molecule is repaired in the nucleus and transcribed by RNA polymerase II to generate two molecules, the 35S RNA and the 19S RNA. The 35S RNA is bifunctional and acts both as the template for viral DNA synthesis by the viral reverse transcriptase as well as a polycistronic mRNA that is translated by host ribosomes to yield all but one of the viral proteins (Sha *et al.*, 1995; Hohn and Fütterer, 1997). The 19S RNA is a monocistronic mRNA that encodes a multifunctional protein (P6) that, among other things, transactivates translation of the 35S RNA and forms cytoplasmic inclusions that act as sites for reverse transcription of the 35S RNA and the formation of new virus particles (Sha *et al.*, 1995; Hohn and Fütterer, 1997; Li and Leisner, 2002).

6.2.2 Cell-to-cell and systemic movement of plant viruses

Most cells within a plant are linked symplastically by a local network of intercellular connections (plasmodesmata) and to cells in other tissues and organs via the phloem: the network of living vascular cells that conduct sugars and other more complex molecules, including RNA and protein molecules, over longer distances through the plant (Roberts and Oparka, 2003; Oparka, 2004). In contrast to the viruses of many other multicellular organisms, plant viruses do not need to physically break out of one host cell in order to invade the next because they can exploit this symplastic transportation network. To be a successful plant pathogen, it is not sufficient for a virus to be able to replicate efficiently in plant cells. For example, the insect virus flock house virus can replicate in plant cells but, unaided, cannot spread out from a primary infected plant cell (Dasgupta *et al.*, 2001). Thus, the ability to exploit the symplastic network effectively must be a strong selective pressure driving the evolution of plant viruses and the genesis of new plant-infecting viruses.

Virus movement, starting from a primary inoculated cell and spreading to other parts of the plant, breaks down into two phases that correspond to its negotiation of the local (plasmodesmal) and systemic (phloem) symplastic network. Cell-to-cell movement through plasmodesmata is relatively slow and is mediated in most cases by viral movement proteins. Movement proteins fall into two broad types (Lazarowitz and Beachy, 1999). The first type, exemplified by the tobacco mosaic virus (TMV) movement protein, facilitates cell-to-cell transfer of viral nucleic acids by interacting with host factors to increase the permeability of the plasmodesmata and by unfolding the secondary structure of the viral nucleic acid (Boevink and Oparka, 2005). However, many viral and host factors affecting movement of viral material through the plasmodesmata have multiple roles and affect not only intercellular trafficking, but also viral replication or propagation of silencing or counter-silencing signals (Section 6.5).

The second type of movement protein, exemplified by those of CaMV and the RNA virus cowpea mosaic virus, forms tubules that obliterate the

internal plasmodesmal structure and which are wide enough for entire viral particles to pass through (Kasteel *et al.*, 1996; Maule and van Lent, 1996). In some cases (e.g. closteroviruses and potyviruses), it appears that protein components of the virion or non-structural viral proteins associated with virus particles in vivo can facilitate the binding of virus particles to plasmodesmata (Peremyslov *et al.*, 2004; Torrance *et al.*, 2006). It is thought that this is followed by a process in which ribosomes of the receiving cell translate viral RNA into protein and co-translationally pull the RNA in through the plasmodesmata (Torrance *et al.*, 2006).

Systemic movement of viruses through the phloem as virus particles (Simon-Buela and García-Arenal, 1999) or as part of nucleoprotein complexes (Talianski *et al.*, 2003) is a faster process than cell-to-cell movement (Leisner *et al.*, 1992; Blackman *et al.*, 1998). As might be expected for a phloem-mediated process, viral systemic movement broadly follows the flow of photosynthetically fixed carbon metabolites from mature (carbon source) tissues to younger, actively growing tissues or developing storage organs (carbon sinks) (Leisner and Turgeon, 1993; Nelson and van Bel, 1997). However, the processes of viral entry into and exit from the phloem are complex and imaging studies of viruses expressing fluorescent marker proteins have revealed that each part of the process involves specific vein types (Blackman *et al.*, 1998). Furthermore, systemic movement depends on activities (or additional functions) of several types of viral protein including coat and movement proteins and proteins that counteract RNA silencing (Blackman *et al.*, 1998; Ding *et al.*, 2004) (Section 6.5). Additionally, host factors in the phloem may associate with coat or other viral proteins to protect virus particles or ribonucleoprotein complexes during transport (Requeña *et al.*, 2006). The ability to move systemically and cell to cell is also limited or promoted by host factors. Those identified in various plants include pectin methylesterases, jacalin-type proteins and glycine-rich proteins (Dorokhov *et al.*, 1999; Chen *et al.*, 2000; Chisholm *et al.*, 2001; Ueki and Citovsky, 2002, 2005). In the case of pectin methylesterase, it was recently proposed that paradoxically this host factor also promotes RNA silencing against viruses (Dorokhov *et al.*, 2006).

6.3 'Classical' or genetically predetermined resistance

6.3.1 The mechanisms of genetically defined resistance

Until the mid-1990s and prior to the discovery of RNA silencing and its roles in plant defence (Sections 6.4 and 6.5), resistance to viruses was considered to be for the most part genetically predetermined. These predetermined mechanisms could be classified broadly into 'passive' or pre-existing mechanisms, and 'active' or inducible processes (Carr and Klessig, 1989).

Passive resistance might result from a lack of one or more necessary host factors for virus replication or movement. Alternatively, it could arise from the presence of physical barriers to inoculation (cell walls, cuticles or leaf hairs) by mechanical wounding or transmission by insects or other vector organisms. In contrast, active mechanisms are triggered by infection with a pathogen. The best studied example is the hypersensitive response (HR). In a virus-induced HR, the virus is restricted to the initially infected cell(s) and this is often, but not always, accompanied by a self-limiting process of programmed cell death of the infected host cells (Wright *et al.*, 2000; Liu *et al.*, 2005). The HR can trigger an enhanced state of resistance in the vicinity of the HR lesion (local acquired resistance, LAR) or throughout the plant (systemic acquired resistance, SAR) (Ross 1961a,b). Plants exhibiting LAR/SAR show decreased susceptibility not only to the specific pathogen that triggered the HR, but also to pathogens in general. For example, SAR resulting from an HR triggered by a fungus provides resistance to viruses and vice versa (Bergstrom *et al.*, 1982; Naylor *et al.*, 1998).

Both passive and active resistance responses are controlled genetically. Passive resistance resulting from physical barriers to infection usually results from the combined influence of many plant genes (Maule *et al.*, 2007). However, resistance to the systemic movement of tobacco etch virus (TEV) in *Arabidopsis thaliana* could be defined as passive resistance and is conditioned by single genes, *RTM1* and *2* (Whitham *et al.*, 2000; Chisholm *et al.*, 2001). Resistance based on a lack of necessary factors for virus replication or gene expression can be defined by single genes. Potyviruses and certain other ss positive sense RNA viruses possess RNAs that have a covalently linked protein (VPg: virus protein genome linked), instead of a conventional cap structure, at the 5′ end. For the viral RNA to be translated efficiently into protein, it is necessary that the VPg interacts with specific translation factors: eIF4E, eIF(iso)4E or eIF4G, depending on the virus. The interaction is so important that it underpins many examples of recessive (*r* gene) resistance to potyviruses in a wide range of plants including important crops and *Arabidopsis*. Mechanistically, resistance occurs because these *r* genes encode variants of translation factors that are unable to interact with the VpG (Nicolas *et al.*, 1997; Leonard *et al.*, 2000, 2004; Moury *et al.*, 2004). In some cases, the product of a resistance gene may inhibit the viral life cycle. For example, the tomato *Tm-1* semidominant resistance gene encodes an approximately 80-kDa polypeptide that inhibits assembly of the replication complex of tomato mosaic virus (ToMV, a virus closely related to TMV) (Ishibashi *et al.*, 2007).

The HR is nearly always conditioned by a single plant gene that enables a plant to recognise and respond against a specific pathogen (Kachroo *et al.*, 2006; Schoelz, 2006). The best studied resistance genes controlling an HR are dominant (*R*) and their modes of action are explainable in terms of a gene-for-gene relationship, that is, a direct or indirect interaction between the R protein and the product of a corresponding avirulence (*Avr*) gene in the pathogen, that has resulted from the co-evolution of the host and virus (reviewed by

Schoelz, 2006). The first antiviral *R* gene to be isolated was *N* which confers HR-type resistance against almost all strains of TMV (Dunigan *et al.*, 1987; Caplan and Dinesh-Kumar, 2006). The full-length N protein contains a Toll interleukin-1 receptor domain at its N-terminus together with a nucleotide-binding site (NBS) domain and a domain of leucine-rich repeats (LRR) (TIR-NBS-LRR). Through alternative splicing during transcription of the *N* gene, a truncated form of N is produced (N^{tr}) which contains the NBS and TIR domains but only part of the LRR (Marathe *et al.*, 2002). The helicase domain of the TMV 126/183-kDa replicase protein acts as the elicitor of the *N*-mediated HR (Marathe *et al.*, 2002). Activation of N is probably aided by the formation of N/N^{tr} multimers and recruitment of an accessory CC-NB-LRR protein, NRG1 (Peart *et al.*, 2005; Mestre and Baulcombe, 2006). Most other antiviral *R* genes also belong to the NBS-LRR class but contain a coiled–coiled domain at their N-terminus (CC-NBS-LRR) rather than a TIR domain (for recent reviews, see Kang *et al.*, 2005; Caplan and Dinesh-Kumar, 2006; Kachroo *et al.*, 2006; Maule *et al.*, 2007).

The mode of action of R proteins is still not fully understood. In many cases, it is unclear whether elicitor recognition occurs via a direct interaction between the elicitor and R protein. One of the best studied antiviral NBS-LRR R proteins is encoded by the potato *Rx* gene. *Rx*-mediated resistance to potato virus X (PVX) and specificity of recognition depends on the presence of specific amino acid residues within the viral coat protein and properties of the Rx LRR domain (Farnham and Baulcombe, 2006). Initiation of defence signalling by interaction between Rx and the PVX coat protein appears to involve a conformational change in the Rx polypeptide, allowing its conversion from an inhibited to an active state (Rairdan and Moffett, 2006). It is not clear if this reorganisation of the Rx protein requires direct interaction with the elicitor (Rairdan and Moffett, 2006). The genes *Tm-2* and *Tm-2*^{2} confer resistance in tomato against strains of TMV and ToMV (Pfitzner, 2006). As with Rx, the LRR domains of these CC-NBS-LRR proteins are required for recognition of the viral elicitor, which in this instance is the movement protein (Lanfermeijer *et al.*, 2003). However, experimental evidence for a direct elicitor–R protein interaction is lacking (Pfitzner, 2006).

Several examples of *R* genes specifying resistance to non-viral pathogens conform to the 'guard' model in which the R protein monitors the status of a host protein that is modified or degraded during pathogen attack. For example, the *Arabidopsis* protein RIN4 is targeted by effector molecules injected into the plant cell by certain pathovars of *Pseudomonas syringae*. This can be detected in different ways by the R proteins RPM1 and RPS2, resulting in HR induction (Axtell and Staskawicz, 2003; Mackey *et al.*, 2003). At least one antiviral R protein appears to act in a guard-like fashion. The *Arabidopsis HRT* gene controls resistance to turnip crinkle virus (TCV) and the elicitor for this interaction is the viral coat protein (Dempsey *et al.*, 1997; Ren *et al.*, 2000; Zhao *et al.*, 2000). The coat protein binds to and inhibits nuclear localisation of a potential host transcription factor TIP (TCV-interacting protein) that must

be present for an HRT-mediated HR to occur and it has been proposed that HRT may guard TIP by activating an HR in response to binding of TIP by the TCV coat protein (Ren *et al.*, 2005). The *HRT* system is further complicated by a requirement for light and/or the presence of salicylic acid (SA; Section 6.3.3) as well as involvement of an additional protein partner, RRT (regulates resistance to TCV), which negatively regulates levels of HRT. Only plants homozygous for the recessive *rrt* allele are normally able to express *HRT*-mediated resistance to TCV (Kachroo *et al.*, 2000; Chandra-Shekara *et al.*, 2006).

Recent work suggests that the mode of action of N may differ in some important respects from other NBS-LRR proteins due in part to the properties of its TIR domain. Remarkably, the TIR domain, previously considered to be a signalling-related domain, appears to be required for association between N and the elicitor sequence within the TMV replicase protein (Burch-Smith *et al.*, 2007). This study utilised novel in vivo imaging techniques to identify the interaction, and Burch-Smith *et al.* (2007) argue from this that in vitro or yeast-based protein–protein interaction assays may not always reveal R protein–elicitor interactions even when they are direct. This work also suggests that it may not always be correct to classify R protein domains as being involved solely in recognition (e.g. LRRs) or signalling (CC or TIR).

6.3.2 Breakage of genetically defined resistance and short-term evolution of viruses

It is often assumed that viruses evolve rapidly because they have small genomes, short generation times and because RNA viruses in particular are replicated by error-prone RdRp enzyme complexes (Section 6.2.1; Lecoq *et al.*, 2004). Additionally, RNA and DNA viruses exhibit recombination (with themselves, other viruses or even with host sequences), and reassortment can take place in mixed infections of viruses with multicomponent genomes. Although these factors provide the variability necessary for evolution to take place, they do not fully explain why some viral strains or isolates come to predominate within a host cell, host plant or host population while others disappear altogether (Roossinck, 2003; Jeger *et al.*, 2006).

Surprisingly, the genetic make-up of virus populations can remain remarkably stable, even in the face of selection pressure exerted by plant *R* genes, and the evolution of resistance-breaking virus strains occurs more slowly than the evolution of resistance-breaking bacterial or fungal pathogens (García-Arenal *et al.*, 2003; García-Arenal and MacDonald, 2003; Lecoq *et al.*, 2004). In a long-term study, García-Arenal and colleagues have followed the evolution of virulence in tobamoviruses (viruses related to TMV) infecting crops of pepper plants harbouring various *L* resistance alleles. *L* genes confer resistance to specific virus pathotypes in a gene-for-gene manner. Changes in viral isolates and population structure were monitored over a period of around 20 years by sequencing of the gene for a viral replicase protein. Surprisingly, viral

population structure, that is, the proportions of the various pathotypes present in the overall virus population, remained remarkably stable over long periods. When they occurred, changes in population structure were rapid and drastic and appeared to be driven more by competition between viral pathotypes within individual plants than by competition between the host and virus (F. García-Arenal, personal communication).

The idea that plant virus evolution may be at least as strongly driven by virus–virus competition as by plant–virus competition is supported by studies of virus population structure and distribution within an individual plant. In one study, a single tree that was chronically infected with the potyvirus plum pox virus (PPV) developed an enormous diversity of RNA sequence variants, some being unique to specific tree branches, and all spatially separated from each other (Jridi *et al.*, 2006). Similarly, strict spatial separation of PPV variants was found for two recombinant versions of PPV that were identical except for the possession of a marker gene encoding either the green fluorescent protein (GFP) or DsRed. Fluorescence microscopy of doubly infected *Nicotiana benthamiana* leaf tissue showed that the 'green' and 'red' PPV variants did not co-infect the same cells (Dietrich and Maiss, 2003). It remains unclear whether the spatial separation of closely related virus strains and variants is due to triggering of highly localised RNA silencing effects, competition for host cells or resources, or to differences in the efficiency of virus of cell-to-cell movement.

6.3.3 Active resistance to viruses: signalling in the HR and SAR

SA is an essential signal for the induction of SAR and the orchestration of events during and following the HR. Increased biosynthesis of SA, most likely synthesised via an offshoot of the shikimate pathway, occurs following an HR, initially in the immediate vicinity of the lesion but later in some cases throughout the plant (Malamy *et al.*, 1990; Métraux *et al.*, 1990; Wildermuth *et al.*, 2001; Huang *et al.*, 2006; Nobuta *et al.*, 2007; Strawn *et al.*, 2007). The importance of SA was demonstrated by the fact that treatment of plants with solutions of SA or aspirin (acetyl-SA) can induce a SAR-like state of enhanced resistance to pathogens even in otherwise susceptible plants and that the HR and establishment of SAR were compromised in transgenic plants unable to accumulate SA due to expression of salicylate hydroxylase (*NahG* transgenics) (White *et al.*, 1983; Gaffney *et al.*, 1993; Mur *et al.*, 1997). However, the relationship between SA and other signals produced during the HR, such as nitric oxide, reactive oxygen species (ROS) and jasmonic acid (JA), is complex and still not fully understood (Kenton *et al.*, 1999; Alvarez, 2000; Mur *et al.*, 2000).

The relationship between SA and JA signalling is particularly intriguing. JA is an oxygenated fatty acid (oxylipin) which is a signal in resistance to certain bacteria, fungi and insects (Ryan, 1990; Reymond and Farmer, 1998; Thaler *et al.*, 2004). During the 1990s, the JA and SA pathways were viewed

predominantly as being mutually antagonistic but pioneering transcript profiling work revealed significant positive and negative crosstalk between the two pathways (Schenk *et al.*, 2000). Other work has shown that JA is synthesised transiently in the earliest stages of a TMV-induced HR and a recent study suggests that JA plays a role in SAR induction, which was until recently considered a JA-independent process (Kenton *et al.*, 1999; Truman *et al.*, 2007).

SA-induced resistance to viruses is mediated in part by a pathway that appears to involve signals transduced through changes in redox or ROS in the mitochondria (Singh *et al.*, 2004) and that is separate from the NPR1-dependent pathway required for SA-induced resistance to fungi and bacteria (Murphy *et al.*, 1999; Wong *et al.*, 2002). This idea is supported by several lines of evidence including observations that resistance to cucumber mosaic virus (CMV), PVX and TMV in tobacco, as well as to turnip vein clearing virus in *Arabidopsis*, can be induced with non-toxic levels of antimycin A or cyanide (Chivasa and Carr, 1998; Wong *et al.*, 2002). Similar findings with respect to the DNA virus CaMV in *Arabidopsis* were reported (Love *et al.*, 2005, 2007a; Gilliland *et al.*, 2006). These chemicals, and SA itself, impede electron flow through the respiratory electron transport chain and enhance ROS levels in the mitochondria (Xie and Chen, 1999; Norman *et al.*, 2004).

Additional support for the existence of a mitochondrial signalling pathway comes from studies using transgenic plants or viral vectors to examine the effects on virus infection of altering levels of alternative oxidase (AOX), an enzyme that negatively regulates mitochondrial ROS levels (Gilliland *et al.*, 2003; Murphy *et al.*, 2004). Increasing the expression or activity of AOX not only compromised resistance but also revealed the existence of a separate pathway activated by SA and potentially involving the induction of the host RNA-dependent RNA polymerase 1 (RDR1) (Gilliland *et al.*, 2003). This is discussed further below and in Sections 6.4 and 6.5. Further work revealed that the 'mitochondrial' pathway for induction of resistance to viruses by SA may not be important for virus resistance in squash (*Cucurbita pepo*) (Mayers *et al.*, 2005). Thus, caution is required when attempting to extrapolate findings on plant–virus interactions from model systems such as tobacco and *Arabidopsis* to other host species.

6.3.4 Mechanisms of virus resistance in the HR and SAR

Cell death during the HR is not essential for resistance to viruses. Evidence for this includes the observation that depletion of oxygen inhibits cell death in tobacco during *N* gene-mediated HR following TMV inoculation, but the virus is still restricted to the vicinity of the primary infection site (Mittler *et al.*, 1996). In restriction of PVX by *Rx* in potato, there is normally no cell death associated with the resistance response (Bendahmane *et al.*, 1999).

Experiments using a variety of plant–virus interactions show that cell death and resistance can be entirely separated at the genetic level (reviewed by Schoelz, 2006). For example, an HR triggered by CMV in cowpea plants

consists of two separate phenomena: resistance to virus replication and the induction of host cell death that are triggered by specific and distinct amino acids within the viral elicitor (in this case viral RdRp; Kim and Palukaitis, 1997). The p19 protein encoded by tomato bushy stunt virus (TBSV) elicits systemic necrosis in *N. clevelandii* and an HR in tobacco. Scholtoff and co-workers showed that it was possible to mutate the p19 gene sequence so that cell death induction in both hosts was abolished but resistance was still triggered in tobacco (Chu *et al.*, 2000). Also, cell death and resistance induction triggered by CaMV in *Nicotiana* species are controlled by separate host genes (Cole *et al.*, 2001).

Studies of *N* gene-mediated HR have shown that TMV remains viable in living cells at periphery of the necrotic lesions. This was revealed directly by fluorescence microscopy of lesions triggered by infection with recombinant TMV expressing the green fluorescent protein (Wright *et al.*, 2000; Murphy *et al.*, 2001). Virus in living cells at the lesion periphery remains infectious and can be rescued from HR lesions for almost 2 weeks following HR formation by shifting the temperature to above 32°C (Weststeijn, 1981). At this temperature, *N* gene-mediated resistance breaks down and the virus spreads out from the HR lesion. However, the mechanisms preventing viral escape from lesions below this critical temperature have not been discovered, nor is it understood how virus infection is inhibited in plant tissues exhibiting LAR or SAR.

Attempts to identify the molecular determinants of resistance in plants that have undergone an HR or are exhibiting SAR led to the discovery of pathogenesis-related (PR) proteins (Gianinazzi *et al.*, 1970; van Loon and van Kammen, 1970) and other factors such as the inhibitor of virus replication (IVR) from *NN* tobacco (reviewed by Loebenstein and Akad, 2006). Although PR proteins were initially suspected to play a role in resistance to viruses, it became apparent that they are predominately antifungal and antibacterial in nature and no PR proteins have so far been found to have clear antiviral activity when expressed constitutively in transgenic plants (Carr and Klessig, 1989; Cutt *et al.*, 1989; Linthorst *et al.*, 1989; van Loon and van Strien, 1999). Nevertheless, since many of the PR proteins are inducible by SA and appear following the induction of defence reactions, they are often used as markers for induced resistance. IVR has not been as extensively investigated as the PR proteins, although constitutive expression of this factor in transgenic plants does provide some protection against virus infection (Akad *et al.*, 2005). These transgenic plants also expressed increased resistance to a fungal pathogen (Akad *et al.*, 2005), suggesting a broader role for IVR in the regulation of resistance to pathogens.

Studies of SA-mediated resistance, induced by an HR or by treatment of plants with exogenous SA, have shown that inhibition of the virus life cycle can occur in replication, cell-to-cell movement or systemic movement (reviewed extensively by Murphy *et al.*, 1999; Singh *et al.*, 2004; Gilliland *et al.*, 2006). SA-induced mechanisms of resistance to viruses are undoubtedly important in expression of the HR and SAR, since *NahG*-transgenic plants are

compromised in virus resistance (Gaffney *et al.*, 1993; Mur *et al.*, 1997). Currently, the greatest challenge for studies of SA-mediated resistance against viruses is to determine the extent to which it operates through RNA silencing (Section 6.4). The impetus for this comes from the possibility that RDR1 contributes not only to SA-induced resistance to TMV, but also to the discovery that certain viral proteins that counter RNA silencing are also able to inhibit SA-induced resistance. The 2b counter-defence protein of CMV allows this virus to evade SA-induced inhibition of replication and cell-to-cell movement in directly inoculated tissues, although SA still inhibits systemic movement of CMV (Naylor *et al.*, 1998; Ji and Ding, 2001). More recently, the potyviral HC-Pro silencing suppressor protein was found to interfere with SA-mediated resistance to viruses and other pathogens (Pruss *et al.*, 2004; Alamillo *et al.*, 2006) (Section 6.5).

SA-independent mechanisms of resistance to viruses also exist and these likely contribute to HR and SAR-type resistance. For example, tobacco plants exposed to non-lethal concentrations of cadmium ions exhibit resistance to systemic virus movement through modification of the levels of callose in the plant vasculature (Ueki and Citovsky, 2002, 2005). Although the role of this resistance mechanism in the HR or SAR has not been established, callose is known to affect the ability of viruses to spread. Another mechanism of resistance to TMV spread that is not dependent on SA signalling but nevertheless appears to be associated with the *N* gene was discovered during studies of transgenic plants expressing a CMV protein. This resistance manifests itself as inhibition of movement by attenuated viruses, such as engineered TMV-derived vectors that normally do not spread well in non-transgenic plants. Movement of these attenuated viruses is enhanced in transgenic plants expressing the CMV 1a protein, allowing them to produce visible HR lesions or spread systemically in plants incubated at 33°C – above the temperature range at which the *N* gene-mediated HR is operative (Canto and Palukaitis, 2002). Although CMV 1a protein is primarily thought of as a component of the viral replicase complex, this work shows that it is also a counter-defence factor.

6.4 RNA silencing

RNA silencing was first observed in experiments to produce transgenic petunias with altered pigmentation (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Subsequently, the phenomenon has been documented in a broad range of organisms, not only plants (Waterhouse *et al.*, 1998) but also including animals (Fire *et al.*, 1991; Ngô *et al.*, 1998; Bahmramian and Zarbl, 1999), algae (Molnár *et al.*, 2007) and fungi (Romano and Macino, 1992). It describes the silencing of gene expression mediated by short RNA (sRNA) species bearing high-sequence homology to the target of silencing and occurs by mechanisms that are highly conserved across kingdoms. These mechanisms have

therefore likely arisen early in evolution. The mechanisms of RNA silencing include repression of mRNA transcription by methylation of genomic loci, post-transcriptional degradation of target RNAs and inhibition of translation of mRNAs (Llave *et al.*, 2002b; Aukerman and Sakai, 2003; Zilberman *et al.*, 2003). Due to the complexity of these pathways, we focus here on aspects related to antiviral defence and viral pathogenesis. For extensive discussion of other aspects of plant RNA silencing, readers are directed to recent reviews by Brodersen and Voinnet (2006) and Vazquez (2006).

6.4.1 Short-interfering RNA-mediated silencing against viruses

In plants, RNA silencing is a crucial antiviral defence mechanism. After invasion of host cells, a virus may activate host RNA silencing which limits viral accumulation. RNA silencing against viruses is mediated by short-interfering (si) RNAs: short [in the range 21–24 nucleotides (nt)], ssRNA species with perfect or near-perfect complementarity to their target sequences within the virus. A number of features commonly observed in plant virus-derived RNAs may trigger antiviral silencing. These include dsRNA (a common replication intermediate of plant viruses), the lack of a 5′ cap or poly(A) tail, or certain structural features (e.g. imperfectly paired hairpin loops) (Molnár *et al.*, 2005). This diversity in structures that trigger RNA silencing allows it to be effective against both RNA and DNA viruses. A model for RNA silencing against viruses, based on evidence from *Arabidopsis* and other organisms, is illustrated in Fig. 6.1

First, viral RNA is cleaved into short dsRNAs called siRNA duplexes (Hamilton *et al.*, 2002; Xie *et al.*, 2004). Cleavage is catalysed by Dicer-like (DCL) enzymes that have RNase III-like activity. The four *Arabidopsis* DCLs generate viral-derived siRNAs of 21 nt (DCL4 and DCL1), 22 nt (DCL2) and 24 nt (DCL3) (Blevins *et al.*, 2006; Deleris *et al.*, 2006). Action of the DCLs appears to be hierarchical, with DCL4 and secondarily DCL2 restricting accumulation of a range of RNA viruses (Blevins *et al.*, 2006; Bouché *et al.*, 2006; Deleris *et al.*, 2006). However, the relative contribution of each DCL to siRNA generation may vary, dependent on the challenging virus (Blevins *et al.*, 2006). The siRNAs generated by DCLs are methylated by a methyltransferase, HEN1, protecting them from degradation (Yang *et al.*, 2006). Next, siRNA duplexes are unwound and a single strand (the primary siRNA) is loaded into an RNA-induced silencing complex (RISC) (Nykänen *et al.*, 2001; Qi *et al.*, 2005). The *Arabidopsis* RISC complex has not been isolated yet. However, it is likely to include an argonaute (AGO) protein, ten of which are encoded by *Arabidopsis* (Baumberger and Baulcombe, 2005). AGO proteins possess a PAZ (PIWI/Argonaut/Zwille) sRNA-binding domain and a PIWI (P-element-induced wimpy testis) RNase H-like domain that confers endonucleolytic (or 'slicer') activity (Baumberger and Baulcombe, 2005; Zhang *et al.*, 2006). The incorporated siRNA directs sequence-specific cleavage of viral RNA by the AGO protein (Baumberger and Baulcombe, 2005; Zhang *et al.*, 2007).

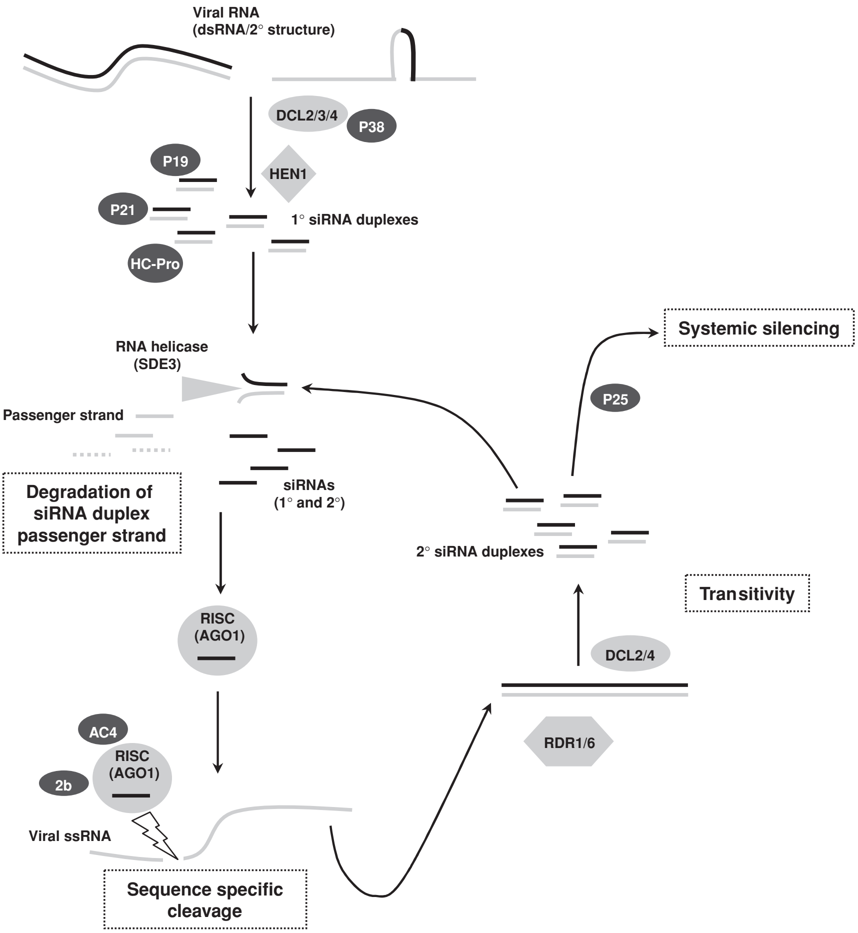

Figure 6.1 siRNA-mediated RNA silencing against viruses. According to this scheme for siRNA-mediated silencing against viruses, dsRNA formed during viral replication, as a consequence of viral gene expression or due to secondary structure, triggers the mechanism. Viral-encoded silencing suppressors are depicted by dark grey ovals containing white lettering, at their probable/confirmed sites of action. Host-encoded components of the silencing machinery are depicted by light grey shapes containing black lettering. DCLs 2, 3 and 4 cleave the dsRNA to form primary siRNA duplexes that are methylated by HEN1. The duplexes are likely unwound by an RNA helicase such as SDE 3 (Dalmay *et al.*, 2001), with each duplex yielding a primary siRNA and a passenger strand. The passenger strand is degraded by unknown mechanisms and the siRNA incorporated into a RISC, one component of which is AGO1. RISC conducts sequence-specific degradation of the source of the dsRNA (i.e. the virus) using the siRNA as a guide. The products of target cleavage feed into the transitivity phase of RNA silencing. In this phase, gene products including RDRs 1 and 6 synthesise further dsRNA from the viral cleavage products (Diaz-Pendon *et al.*, 2007). DCLs 2 and 4 cleave the dsRNA, generating secondary siRNA duplexes from regions beyond the primary site of cleavage. Secondary siRNAs yielded by the secondary siRNA duplexes direct RISC to sequence-specific cleavage of the original source of dsRNA. Furthermore, they act within the systemic signalling pathway of RNA silencing which triggers RNA silencing in distal, uninoculated tissues. RISC, RNA-induced silencing complex.

RNA silencing can be amplified by the process of transitivity. During transitivity, host encoded RDRs synthesise dsRNAs from regions 5′ and 3′ to the primary siRNA cleavage site (Vaistij *et al.*, 2002; Himber *et al.*, 2003). Secondary siRNAs are generated from these dsRNAs. Transitivity results in amplification of RNA silencing, enabling the plant to anticipate and inhibit the replication of the virus (Vaistij *et al.*, 2002; Himber *et al.*, 2003). Secondary siRNAs move across long distances via a relay amplification mechanism and the passage of siRNAs has been detected in the phloem (Himber *et al.*, 2003; Yoo *et al.*, 2004). This suggests that transitivity may contribute to the systemic silencing signal that moves through the phloem and confers viral resistance in distal tissues (Palauqui *et al.*, 1997; Himber *et al.*, 2003).

6.4.2 RNA silencing of endogenous transcripts: micro-RNAs and *trans*-acting siRNAs

Micro (mi) RNAs are 20–24 nt sRNAs encoded by endogenous plant loci that target endogenous mRNAs for RNA silencing (Llave *et al.*, 2002a; Guo *et al.*, 2005). The miRNA loci are separate from those encoding their target mRNAs (Reinhart *et al.*, 2002). miRNAs were first identified in *Caenorhabditis elegans* and have since been found in a range of plants and animals (Lee *et al.*, 1993; Lagos-Quintana *et al.*, 2001; Axtell and Bartel, 2005). miRNAs and their target mRNAs are highly conserved between plant species (Axtell and Bartel, 2005). By a combination of computational and experimental analyses, more than 80 families of miRNAs and a number of their mRNA targets have been identified in *Arabidopsis* (http://asrp.cgrb.oregonstate.edu; Gustafson *et al.*, 2005; Kidner and Martienssen, 2005). Many miRNAs are encoded by more than one locus and target more than one mRNA (Reinhart *et al.*, 2002; Rhoades *et al.*, 2002). A large proportion of the targeted mRNAs encode transcription factors regulating developmental gene expression and hormone response pathways, such as those involved in auxin and abscisic acid signalling (Reinhart *et al.*, 2002; Rhoades *et al.*, 2002; Sunkar and Zhu, 2004; Guo *et al.*, 2005).

The biogenesis of plant miRNAs (Fig. 6.2) is not entirely understood and much has been inferred from animal models (Bartel, 2004). As in animals, plant miRNAs are transcribed from miRNA loci within the plant genome (Aukerman and Sakai, 2003). These transcripts, called pri-miRNAs, are capped, polyadenylated and in plants are typically ~1 kb (Aukerman and Sakai, 2003; Xie *et al.*, 2005). Pri-miRNAs fold back on themselves to produce a hairpin structure. The hairpin structure is formed by base pairing between two regions of complementarity that include the miRNA sequence itself (Reinhart *et al.*, 2002). This structure is processed to yield a pre-miRNA which is ~80–200 nt long (Reinhart *et al.*, 2002; Lee *et al.*, 2003; Kurihara and Watanabe, 2004; Parizotto *et al.*, 2004; Kurihara *et al.*, 2006). In plants, processing of the pre-miRNA takes place in the nucleus and requires DCL1 (Papp *et al.*, 2003; Kurihara and Watanabe, 2004). The miRNA sequence including its complementary, base-paired region is then cleaved out of the hairpin

structure of the pre-miRNA, yielding an miRNA duplex (Bartel, 2004; Kurihara and Watanabe, 2004). Finally, the miRNA duplex is unwound by a helicase and the mature miRNA taken up into an RISC (Llave *et al.*, 2002b). The mature miRNA is used by RISC to direct silencing of specific endogenous plant mRNA molecules, with which it is highly (but not always perfectly) complementary (Llave *et al.*, 2002b; Aukerman and Sakai, 2003). Silencing may occur either through repression of translation of the mRNA or through cleavage of the mRNA (Llave *et al.*, 2002b; Aukerman and Sakai, 2003).

A second class of sRNAs, *trans*-acting (ta) siRNAs, are also depicted in Fig. 6.2. Like miRNAs, tasiRNAs regulate the accumulation of endogenous transcripts (Yoshikawa *et al.*, 2005). tasiRNAs are generated from non-protein-coding endogenous transcripts (Yoshikawa *et al.*, 2005). After an initial cleavage event directed by an miRNA, the transcript is processed by DCL4 to give tasiRNAs (Yoshikawa *et al.*, 2005). The tasiRNAs then direct the cleavage of endogenous transcripts with sequence homology in a process requiring AGO1 (Vazquez *et al.*, 2004).

6.4.3 RNA silencing as a basal resistance mechanism

As discussed earlier, RNA silencing directed against an invading virus causes degradation of viral RNA, limiting viral accumulation in the inoculated leaf and possibly moderating symptom induction (Deleris *et al.*, 2006; Pantaleo *et al.*, 2007). Plants impaired in siRNA-mediated silencing exhibit hypersusceptibility to a range of viruses (Mourrain *et al.*, 2000; Xie *et al.*, 2001; Morel *et al.*, 2002; Bouché *et al.*, 2006; Deleris *et al.*, 2006). Cleavage of a plant virus by RISC was shown to limit cymbidium ringspot virus accumulation, although the involvement of translational inhibition in limiting viral accumulation could not be excluded (Pantaleo *et al.*, 2007). It is conceivable that the action of DCLs on viral RNA may directly limit viral accumulation, but this hypothesis awaits definitive testing (Ruiz-Ferrer and Voinnet, 2007). RNA silencing also acts to restrict unloading of TCV from vascular bundles (Deleris *et al.*, 2006). The mechanism of restriction involves 21-nt siRNAs generated by DCL4 and presumably functions to restrict systemic virus spread within the plant (Deleris *et al.*, 2006).

In animal systems, miRNAs encoded by the host were demonstrated to restrict accumulation of primate foamy virus type-1 (Lecellier *et al.*, 2005). Such a direct antiviral role for miRNAs in plant–virus interactions has not yet been demonstrated. However, flagellin, a PAMP (pathogen-associated molecular pattern) produced by *P. syringae*, induces an miRNA that represses auxin signalling in plant cells (Navarro *et al.*, 2006). The repression of auxin signalling restricts *P. syringae* growth, suggesting that miRNAs do play a role in plant defence against pathogens (Navarro *et al.*, 2006).

Given that RNA silencing is a key pathway in plant antiviral defence, it is not surprising that the ability to perturb or hijack the system has evolved in many viruses. A number of viruses express proteins that

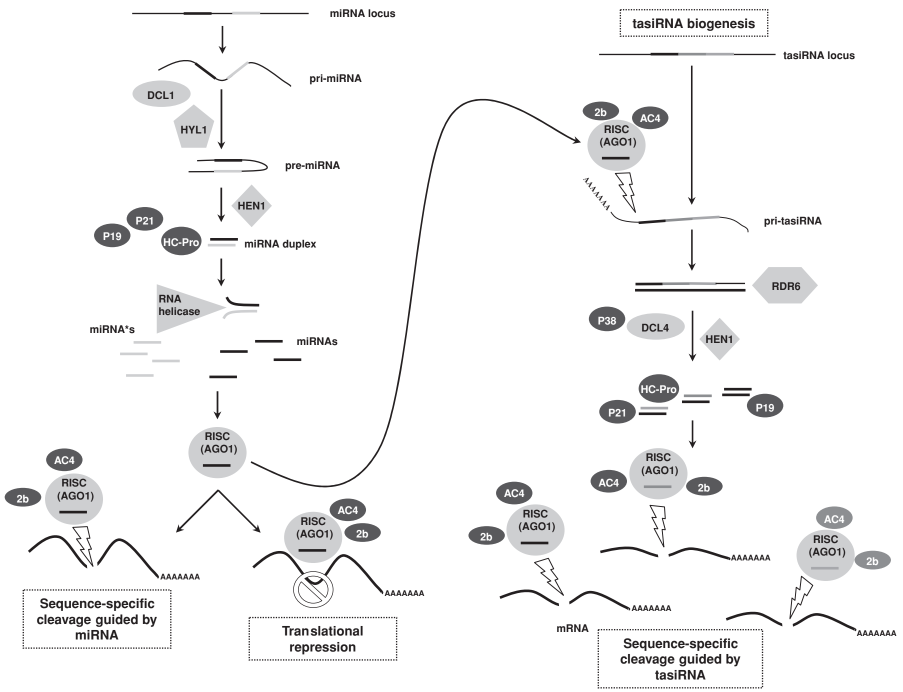

Figure 6.2 miRNA- and tasiRNA-mediated silencing of endogenous transcripts. In this scheme, miRNA-mediated silencing starts with transcription of a pri-miRNA from an miRNA locus. Viral-encoded silencing suppressors are depicted by dark grey ovals containing white lettering, at their probable/confirmed sites of action. Host-encoded components of the silencing machinery are depicted by light grey shapes containing black lettering. The pri-miRNA contains an miRNA sequence (thick dark grey line) and its complementary sequence (thick light grey line). Folding back of the pri-miRNA occurs and processing by the enzymes DCL1 and HYL1 takes place, giving a smaller pre-miRNA. The pre-miRNA is cleaved, leaving only an miRNA duplex consisting of the miRNA and its complementary sequence (the miRNA*). The miRNA duplex is unwound and incorporated into a RISC that includes AGO1. There it is used to target endogenous mRNAs in a sequence-specific manner for translational repression (without degradation) or cleavage. Targeting of tasiRNA transcripts by miRNA–RISC complexes also occurs. In this process, a non-coding pri-tasiRNA is transcribed from a genomic tasiRNA locus. It is then targeted in a sequence-specific manner by a RISC bearing a complementary miRNA. This initiates synthesis of a complementary RNA strand by RDR6, yielding dsRNA, followed with phased cleavage by DCL4 and methylation by HEN1, generating tasiRNA duplexes (Allen *et al.*, 2005). tasiRNAs from the duplexes bear complementarity to endogenous mRNAs and are used to program RISCs to target these mRNAs.

suppress siRNA-mediated silencing and miRNA-mediated silencing, increasing viral accumulation and causing disturbances in plant development (see Section 6.5). Remarkably, some viruses may encode their own siRNAs, for example the dsDNA virus CaMV (Moissiard and Voinnet, 2006). A number of sRNAs bearing near-perfect sequence complementarity to host mRNAs are

generated from CaMV transcripts (Moissiard and Voinnet, 2006). One such sRNA was shown to function in vivo as an siRNA, downregulating a host transcript (Moissiard and Voinnet, 2006).

6.4.4 The phenomenon of plant recovery

Recovery of a plant from a viral infection was reported as early as 1928. In tobacco infected with tobacco ringspot virus, the lower, initially infected leaves exhibited necrotic patches, but the upper leaves were asymptomatic and resistant to secondary infection (Wingard, 1928). The term 'recovery' gained a slightly different meaning in the 1990s when it was applied to certain lines of transgenic plants exhibiting pathogen-derived resistance (van der Vlugt *et al.*, 1992; Lindbo *et al.*, 1993). During this period, expression of viral-derived sequences in transgenic plants was being examined as a potential source of artificial resistance genes (hence 'pathogen-derived'). Plants expressing transgenes derived from TEV or potato virus Y (PVY) recovered from infection with the respective virus, despite being initially susceptible (van der Vlugt *et al.*, 1992; Lindbo *et al.*, 1993). Upper leaves of the plants were asymptomatic and resistant to secondary infection with the same virus (van der Vlugt *et al.*, 1992; Lindbo *et al.*, 1993). For plants expressing TEV-derived transgenes, the resistance was demonstrated to be specific since it did not protect against a closely related virus (Lindbo *et al.*, 1993). The mechanism of virus resistance was found to operate at the transcript level (van der Vlugt *et al.*, 1992; Lindbo *et al.*, 1993). It was later demonstrated that non-transgenic plants exhibited a similar recovery from CaMV and the nepovirus tobacco black ring virus (TBRV) infection, becoming resistant to secondary infection (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). As in pathogen-derived resistance against TEV and PVY, recovery occurred via a mechanism operating at the transcript level and this was shown to be due to RNA silencing (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). The TBRV infected plants were also highly resistant to a recombinant PVX strain harbouring TBRV sequences, indicating that RNA silencing might mediate cross-protection between viral strains that have sequence homology (Ratcliff *et al.*, 1997).

6.4.5 Dark green islands

Certain virus–host combinations may result in mosaic symptoms in which discrete, dark green areas are observed within otherwise yellow or light green (chlorotic) leaves (Atkinson and Matthews, 1967; Loebenstein *et al.*, 1977). The chlorotic leaves have altered histology and reduced photosynthetic capacities compared to leaves of uninfected plants and contain high viral titres (Lehto *et al.*, 2003). By contrast, dark green islands (DGIs) exhibit histology and photosynthetic capacity similar to leaves of uninfected plants (Lehto *et al.*, 2003). They also contain very low viral titres and are resistant to reinfection with the same or closely related viruses but not an unrelated virus (Fulton,

1951; Atkinson and Matthews, 1970; Loebenstein *et al.*, 1977; Suzuki *et al.*, 1989; Moore *et al.*, 2001). DGIs have defined borders, may be round or irregularly shaped, and are observed in a number of host–virus combinations (for review, see Moore and MacDiarmid, 2006). The rate of expansion of DGIs is too rapid to be explained by a single cell becoming resistant and then growing and dividing (Atkinson and Matthews, 1970). This led to the idea that a diffusible signal is responsible for maintenance of DGIs (Atkinson and Matthews, 1970).

Moore and co-workers (2001) demonstrated that DGI formation and maintenance occur by an RNA silencing process much like that responsible for recovery from viral infection (see Section 6.4.4). They showed that tamarillo mosaic virus (TaMV) RNA was undetectable in DGIs on systemically infected *N. benthamiana* plants. A heterologous viral vector based on white clover mosaic virus was developed, harbouring a section of the TaMV genome. Plants that were already infected systemically with the vector were inoculated with TaMV. In yellowed tissue of the super-infected plants, transcripts of both TaMV and the heterologous vector were detected. However in DGIs, transcripts of virus or vector could not be detected. Furthermore, if transgenic plants harbouring a TaMV-derived transgene were infected with TaMV, transgene transcripts were detectable in yellowed tissue and undetectable in DGIs (Moore *et al.*, 2001). These results were supported by the discovery that the potyviral suppressor of RNA silencing, HC-Pro, was able to suppress DGI formation when expressed transgenically in *N. benthamiana* (Yelina *et al.*, 2002). Also, tobacco plants in which the RDR NtRDR1 had been silenced no longer exhibited DGIs when infected with TMV (Xie *et al.*, 2001). High levels of siRNAs against viral RNA are present in DGIs, raising the possibility that these are the diffusible DGI signal (Yelina *et al.*, 2002). siRNAs are able to travel 10–15 cells from the cell in which they were generated (Himber *et al.*, 2003), a distance which may account for the rate of expansion of DGIs (Moore and MacDiarmid, 2006).

6.4.6 RNA silencing and induced resistance

A number of lines of evidence suggest that RNA silencing is involved in SA-induced resistance to viruses (see Sections 6.3.3 and 6.3.4). The 2b protein, a suppressor of gene silencing encoded by cucumoviruses (Section 6.5), also suppresses SA-induced resistance and inhibits upregulation of AOX (Ji and Ding, 2001). Moreover, components of the RNA silencing machinery may be required for SA-induced resistance. RDR1 enzymes identified in *N. tabacum* (NtRDR1), *Arabidopsis* (AtRDR1) and *Medicago trunculata* (MtRDR1) are induced by SA treatment and TMV infection (Xie *et al.*, 2001; Yu *et al.*, 2003; Yang *et al.*, 2004; for a description of past and current RDR nomenclature, see Wassenegger and Krczal, 2006). Silencing or knockout of these enzymes results in hypersusceptibility to some RNA viruses (Xie *et al.*, 2001; Yu *et al.*, 2003; Yang *et al.*, 2004). NtRDR1 is not induced by treatment with antimycin A, leading researchers to propose that SA induces multiple viral resistance

pathways (Gilliland *et al.*, 2003; Singh *et al.*, 2004) (Section 6.3.3). Significantly, *N. benthamiana* encodes a non-functional homologue of NtRDR1, NbRDR1m, which is induced by SA treatment and TMV infection (Yang *et al.*, 2004). Compared to other *Nicotiana* spp., *N. benthamiana* is notable for its hypersusceptibility to many viruses.

A recent study suggests that RDRs may play a role in other induced resistance mechanisms. While examining the interaction between *N. attenuata* and a herbivorous pest, *Manduca sexta*, a homologue of NtRDR1 was cloned (Pandey and Baldwin, 2007). Expression of the homologue, called NaRDR1, increased in response to treatment with SA, JA or a combination of *Manduca sexta* oral secretions and wounding. Plants in which NaRDR1 was silenced were hypersusceptible to *M. sextuca* due to an inability to accumulate direct defence compounds such as nicotine. Consequently, the authors proposed that NaRDR1 is involved in controlling rapid metabolic adjustments in response to herbivore attack (Pandey and Baldwin, 2007). Typically, plants encode numerous RDRs. For example, *Arabidopsis* encodes six (Wassenegger and Krczal, 2006). This raises the possibility that RDR activity could be induced by a number of plant defence elicitors or hormones for the purposes of rapid transcriptional reprogramming.

6.5 Viral suppressors of RNA silencing

RNA silencing is a potent antiviral defence and it is hard to imagine how viruses could persist unless they had evolved the ability to suppress or evade it. Viral-encoded suppressors of RNA silencing (VSRs) probably exist for most, if not all, plant viruses (reviewed by Voinnet, 2005; Li and Ding, 2006). They comprise a variety of viral-encoded proteins that interact with various silencing components at different stages in the silencing pathways. While many VSRs have now been described (Table 6.1), in only a few cases do we know precisely where they interact within the RNA silencing mechanism (Fig. 6.1) or the miRNA/tasiRNA-mediated mRNA regulation pathway (Fig. 6.2). Silencing suppressors have little in common structurally and in most cases also have other functions in the virus infection cycle (Table 6.1). As several of the VSRs are encoded by overprinting of other viral-encoded genes, but read in a different reading frame, it was suggested that the genes for VSRs may have been later additions to the genomes of plant viruses (Li and Ding, 2006). This idea is hard to reconcile with the fact that both plants and animals have related processes and many of the same components are involved in RNA silencing (see Voinnet, 2005; Li and Ding, 2006). Thus, RNA silencing is probably an ancient mechanism of gene regulation and defence. In addition, it is difficult to see how viruses could have initially become established without the presence of VSRs unless ancient viruses predated RNA silencing.

Table 6.1 Viral suppressors of RNA silencing (VSRs) of plant viruses

Virus	VSR	Other biological function(s)	Inhibition	References
PLV	p14			Mérai *et al*. (2005)
BMV	CP	Encapsidation; movement		DeWispelare and Rao (2006)
TCV	p38 (CP)	Encapsidation, movement	Dicer cleavage	Qu *et al*. (2003) and Thomas *et al*. (2003)
BYSV; BYV; CTV; GLRAV-2;	p22; p21; p20, p23, CP; p24;	RNA accumulation; Ecapsidation	(p21; bind siRNA; siRNA-RISC formation)	Reed *et al*. (2003), Lu *et al*. (2004) and Chiba *et al*. (2006)
SPCSV	p22	RNase III		Kreuze *et al*. (2005)
CPMV	Small CP			Liu *et al*. (2004)
CMV; TAV	2b	Movement; anti-SA defence response	AGO1 slicer; siRNA signalling	Brigneti *et al*. (1998), Li *et al*. (1999) and Qi *et al*. (2004)
SBWMV	19 K			Te *et al*. (2005)
BSMV; PSLV	γb	Movement; seed transmission		Donald and Jackson (1996) and Yelina *et al*. (2002)
PCV	p15			Dunoyer *et al*. (2002, 2004)
BWYV; CABYV; PLRV	P0		Degrade slicer	Pfeffer *et al*. (2002)
PVX	p25	Movement; RNA helicase	Signalling	Voinnet *et al*. (2000)
PVY; TEV; TuMV	HC-Pro	Aphid transmission; proteinase; movement	Stability of siRNA; siRNA-RISC formation	Anandalakshmi *et al*. (1998), Brigneti *et al*. (1998), Kasschau and Carrington (1998) and Kasschau *et al*. (2003)
RYMV	P1	Movement; accumulation		Voinnet *et al*. (1999) and Hamilton *et al*. (2002)
TMV; ToMV	126 K	RdRp; movement		Kubota *et al*. (2003) and Ding *et al*. (2004)
TRV	16 K	Seed-transmission; RNA accumulation		Reavy *et al*. (2004) and Liu *et al*. (2002)
CymRSV; TBSV	p19	Movement	Signalling; si-RNA-RISC formation	Voinnet *et al*. (1999), Qiu *et al*. (2002) and Havelda *et al*. (2003)
ACLSV	p50	Movement		Yaegashi *et al*. (2007)
TYMV	p69	Movement		Chen *et al*. (2004)
GVA	p10			Chiba *et al*. (2006) and Zhou *et al*. (2006)
RHBV	NS3			Bucher *et al*. (2003)
TSWV	NSs	Movement		Takeda *et al*. (2002) and Bucher *et al*. (2003)

Table 6.1 (Continued)

Virus	VSR	Other biological function(s)	Inhibition	References
RDV	Pns10			Cao *et al*. (2005)
TLCV; TYLCCNV Y10β; ACMV; EACMV; ICMV; MBYV	C2; βC1; AC2; AC4	Activation of virus gene expression	siRNA binding to RISC; adenylate kinase (ADK)	Van Wezel *et al*. (2002), Selth *et al*. (2004), Vanitharani *et al*. (2004), Chellappan *et al*. (2005), Cui *et al*. (2005), Trinks *et al*. (2005) and Wang *et al*. (2005)
BCTV	L2	Activation of virus gene expression	ADK	Wang *et al*. (2005)
CaMV	P6	Translational transactivator; matrix for replication and encapsidation	Dicer cleavage	Love *et al*. (2007b)

[1]Virus abbreviations: (1) Positive-strand RNA viruses: genus *Aureusvirus*, species *Pathos latent virus* (PLV); genus *Bromovirus*, species *Brome mosaic virus* (BMV); genus *Carmovirus*, species *Turnip crinkle virus* (TCV); genus *Closterovirus*, species *Beet yellow stunt virus* (BYSV), *Beet yellows virus* (BYV), *Citrus tristeza virus* (CTV), *Grapevine leafroll-associated virus-2* (GLRAV-2); genus Crinivirus, species *Sweet potato chlorotic stunt virus* (SPCSV); genus *Comovirus*, species *Cowpea mosaic virus* (CPMV); genus *Cucumovirus*, species *Cucumber mosaic virus* (CMV), *Tomato aspermy virus* (TAV); genus *Furovirus*, species *Soil-borne wheat mosaic virus* (SBWMV); genus Hordeivirus, species Barley stripe mosaic virus (BSMV), Poa semilatent virus (PSLV); genus *Pecluvirus*, species *Peanut clump virus* (PCV); genus *Polerovirus*, species *Beet western yellows virus* (BWYV), *Cucurbit aphid-born yellows virus* (CABYV), *Potato leafroll virus* (PLRV); genus *Potexvirus*, species *Potato virus X* (PVX); genus *Potyvirus*, species *Potato virus Y* (PVY); *Tobacco etch virus* (TEV); *Turnip mosaic virus* (TuMV); genus *Sobemovirus*, species *Rice yellow mottle virus* (RYMV); genus *Tobamovirus*, species *Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV); genus *Tobravirus*, species *Tobacco rattle virus* (TRV); genus *Tombusvirus*, species *Cymbidium ringspot virus* (CymRSV), *Tomato bushy stunt virus* (TBSV); genus *Trichovirus*, species *Apple chlorotic leaf spot virus* (ACLSV); genus *Tymovirus*, species *Turnip yellow mosaic virus* (TYMV); genus *Vitivirus*, species *Grapevine virus A* (GVA). (2) Negative-strand RNA viruses: genus *Tenuivirus*, species *Rice hoja blanca virus* (RHBV); genus *Tospovirus*, species *Tomato spotted wilt virus* (TSWV). (3) Double-stranded RNA virus: genus *Phytoreovirus*, species *Rice dwarf virus* (RDV). (4) Single-stranded DNA viruses: genus *Begomovirus*, species *Tomato leaf curl virus* (TLCV), *TLCCNV-Y10 DNA* β, *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *Indian cassava mosaic virus* (ICMV), *Mungbean yellow mosaic virus* (MYMV), *Tomato golden mosaic virus* (TGMV); genus *Curtovirus*, species *Beet curly top virus* (BCTV). (5) Double-stranded DNA virus: genus *Caulimovirus*, species *Cauliflower mosaic virus* (CaMV).

6.5.1 Detection of silencing suppressors

VSRs were initially described as pathogenicity enhancers, either of their own genomes or those of other viruses (Vance *et al.*, 1995; Pruss *et al.*, 1997; Li *et al.*, 1999). This was seen in assays involving expression of the proteins from their own genomes, the genomes of heterologous viruses (viral-based expression vectors) or from transgenes. Expression of these proteins in transgenic plants silenced for expression of reporter genes (GFP or GUS) led to loss of the

silencing of the reporter genes. Through this approach, the first two silencing suppressors were described – the helper component/proteinase (HC-Pro) encoding sequences of the potyviruses TEV and PVY and the 2b protein of the cucumovirus CMV (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998). Silencing suppression was confirmed using two different assays with the same or similar proteins. That is, transgenic expression HC-Pro of TEV was shown to suppress transgenic silencing of GUS, while the expression of PVY HC-Pro from a PVX expression vector was able to suppress the induced silencing of a GFP transgene. Similarly, the CMV 2b protein also suppressed RNA silencing in both assays (Brigneti *et al.*, 1998; Guo and Ding, 2002). Initially, both assays were used interchangeably. This led to many proteins being assessed in the more rapid PVX-expression assay, which not only identified numerous VSRs but also appeared to indicate that some viruses (including PVX) did not have VSRs, although other potexviruses did have silencing suppressors (Voinnet *et al.*, 1999). In the case of PVX, it was discovered that the p25 movement protein was a silencing suppressor but that it did not function in suppressing silencing that was already established (Voinnet *et al.*, 2000). This type of activity was not revealed by silencing suppression assays based on virus expression-vectors. Therefore, a third assay was developed, described as the agro-patch assay (Voinnet *et al.*, 2000) in which agroinfiltration was used to co-infiltrate transgenic plants expressing GFP with a plasmid transiently expressing the VSR along with one transiently expressing an inducer of gene silencing (such as sense or antisense GFP). A variation of this technique that did not require transgenic plants was to use agroinfiltration to transiently co-express the GFP transgene, the putative suppressor and a dsRNA hairpin construct of GFP to induce RNA silencing (Johansen and Carrington, 2001).

At the time of these early experiments, none of the components of the RNA silencing machinery had been described. Thus, VSRs were differentiated based on whether they were able to suppress the maintenance of RNA silencing in leaves that had already been silenced (such as the potyvirus HC-Pro protein) or whether they suppressed only initiation of RNA silencing in leaves that were not silenced (such as the CMV 2b protein and the PVX p25; Brigneti *et al.*, 1998; Voinnet *et al.*, 2000). The CMV 2b VSR also prevented long-distance signalling of silencing (Guo and Ding, 2002), although the mechanism of this is still not clear. To distinguish between local initiation of RNA silencing and the initiation of RNA silencing in upper leaves that follows the transport of the silencing signal, these steps are now referred to as cell autonomous and cell non-autonomous silencing, respectively, and VSRs are now described according to which of these steps they affect (see Voinnet, 2005; Li and Ding, 2006). In one instance, a VSR was not able to suppress silencing in any of the above assays, but did block the initiation of long-distance or systemic silencing. This was assayed in a GUS-expressing scion grafted onto a transgenic rootstock co-expressing the viral-encoded protein along with the self-silencing reporter transgene (GUS) (Lu *et al.*, 2004). Here, the VSR

functioned by preventing the silencing signal in the rootstock from activating silencing in the scion. A suppression of RNA silencing assay was also developed using cells of *Drosophila melanogaster* instead of plants leaves (Reavy *et al.*, 2004). Drosophila cells growing in tissue culture were transfected simultaneously with three plasmids: one expressing the β-galactosidase (lacZ) gene, one a dsRNA homologous to part of the lacZ gene and one the putative VSR. This allowed detection of the activity of the VSR in a cellular colorimetric assay. PVY HC-Pro, TRV 16 K and groundnut rosette virus ORF3 (M. Taliansky, personal communication) proteins suppressed RNA silencing in this assay, but the CMV 2b protein could not.

6.5.2 Properties of VSRs

Currently, more than 40 VSRs have been characterised (Table 6.1). Many VSRs described have other functions that were already known. This is particularly true for the HC-Pro proteins of potyviruses that facilitate aphid transmission of these viruses as well as proteolytic processing of the polyprotein expressed from the genomes of these viruses (Rajamäki *et al.*, 2004). HC-Pro is also involved in virus movement, although whether this is a property distinct from its function in silencing suppression is not clear. The p19 of TBSV and the 2b proteins of CMV as well as the related cucumovirus tomato aspermy virus (TAV) may also have dual functions as VSRs and determinants of virus movement. In the example of CMV 2b protein, this protein was first identified as a factor needed for virus systemic movement and only later found to be a VSR (Ding *et al.*, 1995). Possibly, the ability of the 2b protein to facilitate movement may be a manifestation of its ability to inhibit RNA silencing suppression or resistance mediated by SA (Ji and Ding, 2001; M. Lewsey, unpublished data). However, in the case of the p25 of PVX, the relationship between its roles as a VSR and a movement protein is unequivocal since the suppression of local silencing by this protein is a prerequisite for the cell-to-cell movement (Bayne *et al.*, 2005). For several viruses, including those possessing a block of three movement protein genes, one of these, a cysteine-rich protein, has silencing suppression activity (SBMV 19 K, BSMV γb, PLV p15, TRV 16 K; Table 6.1). These three examples are the only VSRs that appear to be similar to each other in their protein structures.

Some VSRs can determine symptom severity and promote virus multiplication and spread even when expressed by a vector derived from a heterologous virus and this has been used as an indirect assay for VSR activity. Hence, deletion of the TRV 16 K protein gene prevented systemic movement of the virus but movement was restored by insertion of the CMV 2b gene, the barley stripe mosaic virus (BSMV) γb gene or the soilborne wheat mosaic virus 19 K gene (Liu *et al.*, 2002). Later studies verified that both the BSMV γb and TRV 16 K proteins were VSRs (Yelina *et al.*, 2002; Reavy *et al.*, 2004). Also, the BSMV γb protein could be complemented by HC-Pro, which then suppressed interference produced by RNA silencing

between PVX and TMV, each carrying GFP sequences. Such complementation studies demonstrated differences in host-specific functions of these proteins, presumably reflecting other roles of the VSRs besides suppressing RNA silencing. For example, substitution of the HC-Pro gene of wheat streak mosaic virus (WSMV) by those of other tritimoviruses (several WSMV isolates and oat necrotic mottle virus, ONMV), rymoviruses (agropyron mosaic virus and hordeum mosaic virus) or potyviruses (TEV, TuMV) supported infection of wheat by the modified WSMV. However, of these only HC-Pro from WSMV/ONMV supported infection on oats and maize (Stenger and French, 2004). This result revealed that the properties of a VSR can control host range.

While many VSRs are pathogenicity enhancers, not all viral-encoded proteins that enhance pathogenicity are VSRs. Examples of non-VSR pathogenicity enhancers include the Nib- and NIa-expressing sequences of PVY (Brigneti *et al.*, 1998; Germundsson *et al.*, 2007). Meanwhile, not all VSRs induce symptoms: while the 2b genes of CMV and TAV enhance pathogenicity, the 2b gene of the ilarvirus tobacco streak virus did not. More recently, it was proposed that the 2b protein of CMV does not directly enhance symptom expression in *Arabidopsis*. Diaz-Pendon *et al.* (2007) suggest that early in infection the 2b protein inhibits symptom development, whereas later 2b-mediated pathogenicity enhancement results from induction of host genes controlling the silencing pathway. This contrasts with the more widely held view that the differences in the ability of various VSRs to induce symptoms is directly related to their ability or inability to interfere with the processing or targeting of miRNAs (Kasschau *et al.*, 2003; Chapman *et al.*, 2004; Chen *et al.*, 2004; Dunoyer *et al.*, 2004). This interpretation has also been used to explain differences in symptom induction between 2b proteins from mild versus severe strains of CMV (Zhang *et al.*, 2006; Lewsey *et al.*, 2007).

6.5.3 Interactions of specific VSRs with the RNA silencing pathway

Modes of action have been determined only for a few VSRs. Since these proteins are multifunctional, mutating the viral genes encoding VSRs often results in pleiotropic effects, which complicates defining their modes of action. Thus, while the role of the HC-Pro protein in aphid transmission of potyviruses is separate from its role in silencing suppression, other activities may be inextricably linked. For example, the seed transmission function of the BSMV γb and TRV 16 K proteins may involve silencing suppression. Similarly, the roles of many VSRs in virus replication and movement might be related specifically to silencing suppression (Bayne *et al.*, 2005; Takeda *et al.*, 2005) or suppression of other host defence responses (Ji and Ding, 2001; Alamillo *et al.*, 2006).

An additional complication is that some viruses encode more than one suppressor protein. For example, the closterovirus citrus tristeza virus (CTV)

encodes three VSRs, two of which (p20 and p23) function in the agro-patch assay or when expressed in transgenic plants carrying a silenced GUS gene. Only p20 and the third VSR (the coat protein) inhibited systemic movement of the silencing signal in grafted transgenic plants (Lu *et al.*, 2004). These three VSRs must therefore act in different but overlapping ways to inhibit silencing and promote CTV infection.

Since two VSRs, P19 and HC-Pro, were found to be RNA-binding proteins, it seemed possible that this might be the general mode of action. A number of VSRs were tested for the ability to bind ds siRNAs or longer dsRNAs. Indeed, most of the VSRs tested were able to bind one or both sets of dsRNAs (Mérai *et al.*, 2006). Two notable exceptions were the 2b proteins of CMV and TAV. However, since the assay was based on the VSRs being present in the soluble phase of plant extracts and the cucumoviral 2b proteins are associated with the nucleus, it seems likely that these proteins were not present in the soluble protein extracts tested. Currently, partly due to technical limitations and the relatively limited number of VSRs for which mechanistic information is available, it is difficult to generalise about the modes of action for VSRs. Thus, it is helpful to examine in detail what is known for the small number of relatively well-characterised proteins.

Tombusvirus p19. The crystal structures of p19 VSR of cymbidium ringspot virus and carnation Italian ringspot virus have been determined (Vargason *et al.*, 2003; Ye *et al.*, 2003). The structures show that p19 forms a complex with an siRNA duplex, revealing mechanisms by which p19 can select appropriately sized siRNAs and prevent subsequent interactions. In a heterologous system (*Drosophila*), this resulted in inhibition of interaction between the target siRNA molecules and the RISC, although it did not affect preformed siRNA–RISC complexes (Lakatos *et al.*, 2004). However, this was not the case in plants where p19 apparently prevented the systemic movement of the 21- and 22-nt siRNAs (Havelda *et al.*, 2003), thus facilitating systemic infection of the virus (Fig. 6.1).

Cucumovirus 2b. The CMV 2b protein bound dsRNAs of 25 nt or longer (unpublished data cited by Li and Ding, 2006) and the TAV 2b protein were found to be associated with dsRNAs (unpublished data cited by Diaz-Pendon *et al.*, 2007). In addition, TAV 2b protein was able to bind long ssRNAs in vitro (B. Shi and P. Palukaitis, unpublished data). Since CMV 2b prevented the systemic signalling of RNA silencing, it was thought that it might function by destroying or destabilising the siRNAs or by preventing the siRNAs from binding to the RISC (Guo and Ding, 2002). However, as CMV 2b has also been shown to suppress silencing in protoplasts (Qi *et al.*, 2004), it must have an additional role that affects the establishment of RNA silencing and is unrelated to preventing the extracellular movement of the silencing signal. The recent observation that the CMV 2b binds to and inhibits the function of the slicer component AGO1 of the RISC (Zhang *et al.*, 2006) suggests a direct role for the 2b protein in inhibiting RNA silencing (Fig. 6.1) that appears to be auxiliary to its role in binding siRNAs.

Potyvirus HC-Pro. The potyviral HC-Pro was shown to interfere with production of 21-nt siRNAs by Dicer, but not the 24-nt siRNAs (Mallory *et al.*, 2002; Dunoyer *et al.*, 2004). In addition, HC-Pro bound to siRNAs (Mérai *et al.*, 2006). HC-Pro may reduce the stability of the 21- and 22-nt RNAs generated by Dicer, since methylation of the 3′ ends of these siRNAs was reduced by the HC-Pro while HC-Pro affected much less the methylation of the 24-nt siRNA (Ebhardt *et al.*, 2005). The binding of siRNAs by HC-Pro may also affect the ability of these RNAs to interact with the RISC (Fig. 6.1).

Carmovirus p38. The expression of the TCV p38 completely inhibited the production of siRNAs in the RNA silencing suppression assays. The p38, which is also the capsid protein of TCV, bound to long ss- as well as dsRNAs and to siRNAs. Its ability to bind to long dsRNAs indicates that it may inhibit the cleavage of dsRNAs by Dicer (Fig. 6.1). Specifically, cleavage by DCL4 was inhibited resulting in a loss of accumulation of 21-nt siRNAs, but cleavage by DCL2 was not inhibited, thus producing 22-nt siRNAs (Deleris *et al.*, 2006).

Potexvirus p25. The p25 of PVX affected accumulation of 24-nt siRNAs but less so 21-nt siRNAs (Hamilton *et al.*, 2002). As such, p25 is thought to affect the production of the mobile-targeting signal (Voinnet *et al.*, 2000). Since p25 also influenced local movement, p25 likely also blocks the function of the 21-nt siRNA in adjacent cells as well as systemically (Fig. 6.1).

Closterovirus p21. The structure of the p21 of beet yellows virus has been determined (Ye and Patel, 2005). p21 bound both siRNAs and longer dsRNAs. Since p21 is required for accumulation of viral RNAs in single cells (Peremyslov *et al.*, 1998), binding of siRNAs by p21 may prevent the formation of the siRNA-RISC.

Geminivirus AC2/AC4/L2. The AC4 VSR of geminiviruses did not bind to ds siRNAs but bound to ss siRNAs and miRNAs (Chellappan *et al.*, 2005). Thus, one role for AC4 in RNA silencing suppression is to inhibit the RISC activity after siRNA binding (Fig. 6.1). By contrast, the AC2 VSR of geminiviruses functioned as a transcription factor and activated transcription of a potential negative regulator of RNA silencing (Trinks *et al.*, 2005). AC2 may also bind to DNA (Bisaro, 2006). On the other hand, other geminivirus AC2 homologues including L2 interacted with and inhibited the function of an adenylate kinase which may interfere with the methylation-dependent gene silencing of the geminivirus genomic DNA (Wang *et al.*, 2005).

Polerovirus P0. The P0s of poleroviruses were identified as VSRs (Pfeffer *et al.*, 2002). Their silencing suppressor activities correlated tightly with interaction with a plant orthologue of SKP1, a key component of an E3 ubiquitin ligase complex (SCF or SKP1/Cullin1/F-box/RBX1) (Pazhouhandeh *et al.*, 2006). E3 ubiquitin ligases mediate ubiquitination and subsequent degradation of cellular proteins. The F-box proteins in this complex conferred target specificity of the SCF E3 ubiquitin ligase. P0 was shown to contain a conserved F-box motif that was required for VSR activity of P0. It has been suggested that the role of P0 in suppressing RNA silencing might be by targeting key

components of RNA silencing pathways for degradation (Pazhouhandeh *et al.*, 2006).

6.6 Concluding remarks

The fundamental biology of plant–virus interactions differs from that of other plant–pathogen interactions. It was proposed that this unique dynamic would drive the evolution of virus pathogenicity and plant defence in directions not seen in interactions of plants with cellular or multicellular parasites (Section 6.1). This appears not to be borne out when we examine HR-type resistance to viruses conditioned by dominant *R* genes (Section 6.3) since these R proteins do not have particular properties that distinguish them from R proteins controlling resistance to bacterial or fungal pathogens. Also, the SA and JA signalling systems these R proteins trigger operate in resistance to a wide range of pathogens. However, the resistance mechanisms R proteins activate against viruses may be different from those that protect plants from non-viral pathogens. Some types of *R* gene that control restriction of virus spread (e.g. RTM 1 and 2) or *r* genes encoding variants of host factors required for the virus life cycle target vulnerable points in specific viral life cycles. Finally, RNA silencing emerges as one of the strongest and probably most ancient drivers of virus–plant co-evolution. It may also affect competition between viruses inside the host, leading to the evolution of new viral strains (Section 6.3.2). The unique intimacy between virus and host cell has likely made viruses particularly sensitive to RNA silencing as a resistance mechanism (Section 6.4). In turn, viral manipulation of RNA silencing pathways by VSRs allows viruses to evade this form of resistance and influence plant development (Sections 6.4 and 6.5). Whether or not the manipulation of plant development provides some form of selective advantage to viruses remains to be seen.

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Chapter 7

MARSHALLING THE TROOPS: INTRACELLULAR DYNAMICS IN PLANT PATHOGEN DEFENSE

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Abstract: Plants have evolved sophisticated defense systems against pathogen infection. Initiation of induced defense signaling involves recognition of invading pathogens at the plant cell surface, followed by rapid transcriptional reprogramming and focused transport and delivery of antimicrobial products to the site of infection. This review chapter summarizes recent findings of the importance of intracellular dynamics in the plants defense against microbial pathogens. We first discuss events at the cell wall and apoplast, the 'front line' of defense against the invader. It is clear that some defense peptides and molecules secreted by the plant cell host have antimicrobial activity – until recently relatively little was known about the regulation and coordination of directed accumulation to sites of attempted pathogen attack. Molecular genetic studies on the breakdown of resistance to non-adapted fungi have highlighted the role of proteins involved in exocytic vesicle fusion and their possible regulation by transmembrane MLO proteins. Another key process in perception and defense at the cell periphery in eukaryotes is ligand-stimulated endocytosis of receptors for microbial proteins; an example in plants is the flagellin receptor FLS2. The second part of this chapter focuses on the role of nucleocytoplasmic trafficking across the nuclear pore complex in plant innate immunity. Current work highlights the dynamic regulation of defense proteins and the role of specific components of the nuclear pore complex and the nuclear import and export machinery in response to pathogens. The unexpected recent discovery of NB-LRR immune receptors from different species in the nucleus points to a condensed and rapid pathway of communication between the site of perception and activation of defense genes. While the details on spatial and temporal control of these dynamic intracellular processes remain to be elucidated, it is currently an area of intense research.

Keywords: nucleocytoplasmic transport; exocytosis; receptor endocytosis; cytoskeletal rearrangement; vesicular trafficking; antimicrobial secretion

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7.1 Introduction

Plants have evolved a multilayered immune system to defend against potentially infectious pathogens. Unlike vertebrates, they lack specialized circulatory cells, a somatic adaptive immune system and immunological 'memory', except at the scale of evolutionary time (Jones and Dangl, 2006). Therefore, plants rely on a large repertoire of preexisting immune receptors in probably almost every cell to trigger local and systemic innate immune responses upon infection (Chisholm *et al.*, 2006). The innate immune system is considered to be multilayered because it involves 'preinvasion immunity', expressed principally at the plant surface and 'postinvasion immunity', where virulence factors of adapted pathogens are recognized in a microbial strain/plant cultivar-specific manner. In nature, most plants are resistant to most potential pathogens. Nonhost resistance occurs when all isolates of a microbe are nonpathogenic on a particular host. Often, only plants of a single genus are hosts for a particular pathogen; this is the case for many powdery mildew, rust and bacterial pathogens (Thordal-Christensen, 2003). Resistance to a nonadapted pathogen can be the consequence of preformed barriers or a lack of signals from the plant to induce spore germination or express essential pathogenicity genes; however, it is increasingly evident that many instances of nonhost resistance rely on active cellular defenses. Recent work has shown that resistance to inappropriate fungal pathogens depends on at least two layers of defense: the first, preinvasive, is expressed at the cell wall and apoplastic space to prevent penetration at attempted pathogen entry sites, whereas the second postinvasive layer mediates resistance to fungi that have successfully penetrated, and often results in a localized suicide of cells at the site of infection (termed the hypersensitive response, HR). The dissection of these cooperative layers of nonhost resistance has revealed that dynamic intracellular processes play an important role in both pre- and postinvasive defense.

Preinvasion immunity involves the perception of pathogen-associated molecular patterns (PAMPs) that are highly conserved molecules shared by many microbes. Examples of PAMPs include lipopolysaccharide, a major membrane component of gram-negative bacteria, and flg22, a 22-amino acid peptide epitope conserved in the N terminus of flagellin, the subunit of bacterial surface structure flagellum (Nürnberger *et al.*, 2004; Zipfel and Felix, 2005). Recognition of PAMPs by extracellular receptor-like kinases promptly triggers defense responses that require signaling through mitogen-activated protein kinase (MAPK) cascades and transcriptional reprogramming mediated in part by plant WRKY transcription factors (Asai *et al.*, 2002; Eulgem, 2006). Pathogen perception results in the rapid mobilization of antimicrobial compounds, reactive oxygen species (ROS), actin filament rearrangement and cell wall thickening at the site of attempted invasion (Fig. 7.1).

The second layer of defense acts postinvasively and is termed effector-triggered immunity. Successful pathogens have evolved effector molecules

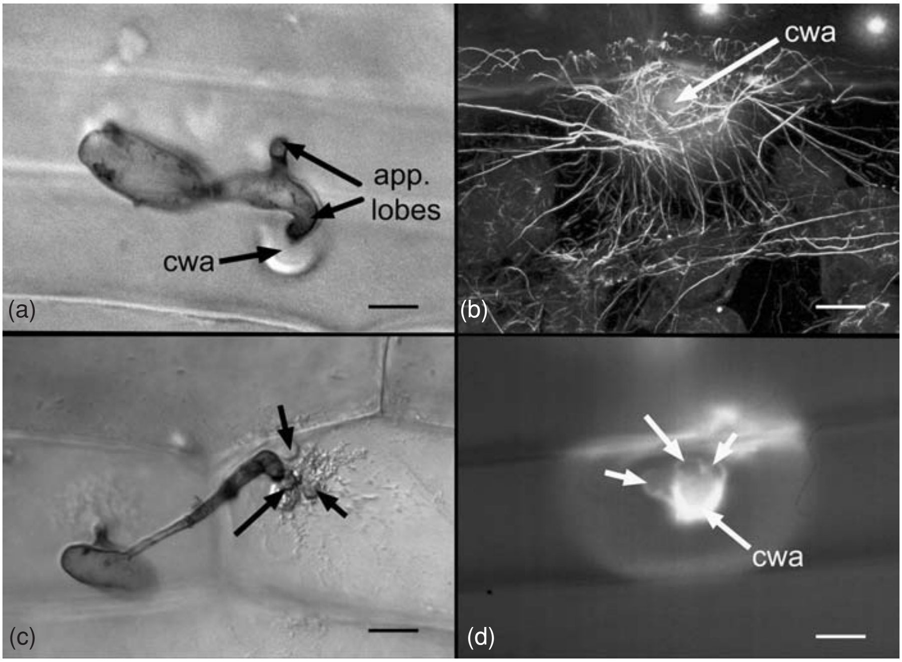

Figure 7.1 Cell wall-associated penetration resistance of barley to *Bgh*. All micrographs represent a view down on the surface of the leaf. (a) A fungal germline showing two appressorial lobes that failed to penetrate a barley epidermal cell, accompanied by cell wall thickening at 48 h after inoculation. (b) Actin filament (fluorescently labeled) with reorganization around a papilla at 20 h after inoculation of barley with *Bgh*. (c) Unsuccessful penetration attempt of *B. graminis* f.sp. *tritici* on barley, accompanied by focal accumulation of vesicle-like bodies containing ROS. Structures are stained by 3,3-diaminobenzidine for H_2O_2. (d) Yellow autofluorescence of phenolic compounds in a cwa and in vesicle-like bodies directly attached to it at 24 h after inoculation. cwa, cell wall apposition; app. lobes, appressorial lobes. Reprinted with permission from Hückelhoven (2007), © 2007 by Annual Reviews, www.annualreviews.org.

that target multiple host proteins to evade or suppress basal resistance to promote virulence. Pathogenic bacteria deliver effectors across the host plasma membrane (PM); little is known about how fungal and oomycete effectors are delivered to the cell, although some are released in the apoplast to counteract host antimicrobial compounds (Chisholm *et al.*, 2006). Effector-mediated resistance is triggered by immune receptors encoded by *Resistance* (*R*) genes. The receptors recognize effectors either directly or by detection of the effector's virulence activity on host cellular targets – according to the 'guard hypothesis' (Dangl and Jones, 2001). Most cloned *R* genes encode intracellular receptors with a C-terminal leucine-rich repeat (LRR) domain and central nucleotide-binding site (NB). The NB-LRR class of R protein can be further divided into coiled-coil (CC) NB-LRR and Toll-interleukin-1 receptor

(TIR) NB-LRR according to their N-terminal domain. The N terminus influences the requirement for downstream defense-response components (Aarts *et al.*, 1998). The recognized cognate effector of any R protein is termed an avirulence (Avr) protein; this pairwise association has been characterized genetically as gene-for-gene resistance, although one R protein can guard multiple effector targets (Marathe and Dinesh-Kumar, 2003). Once an R protein is triggered, resistance is activated, resulting in the initiation of defense signaling, HR and inhibition of pathogen growth. Besides the HR, many of the responses to inappropriate pathogens (nonhost resistance), virulent pathogens (basal resistance) and avirulent pathogens (R-protein-mediated resistance) overlap. Indeed, effector-triggered immunity has been described as a faster and stronger version of basal resistance (Tao *et al.*, 2003; Jones and Dangl, 2006). The local defense response also primes uninfected systemic cells against a broad spectrum of pathogens in a process called systemic acquired resistance (SAR) (Durrant and Dong, 2004). SAR is dependent on the defense-signaling molecule salicylic acid (SA) and on the key regulator NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1). PAMP- and R-protein-dependent responses both result in the induction of *Pathogenesis-Related* (*PR*) gene expression. *PR* genes encode a diversity of peptides, some of which have antimicrobial properties (van Loon *et al.*, 2006).

After expression of defense genes, the cell must deliver and concentrate antimicrobial products to the site of infection. Also, the transcriptional reprogramming of the cell must be tightly controlled to achieve rapid triggering of the defense response while avoiding inappropriate activation. These goals are achieved through regulated directional transport and partitioning of defense regulators between organelles and across membranes. The purpose of this chapter is to review our recent knowledge about these dynamic cellular responses to pathogens, based on current molecular and genetic studies on the intracellular marshalling of defense regulators.

7.2 The front line – exocytosis, endocytosis and vesicle transport

Unlike many mammalian pathogens, potential microbial pathogens of plants remain outside the host cell – bacteria live in the apoplastic space whereas biotrophic fungi and oomycetes form special feeding structures (haustoria) that facilitate parasitism by intimate association with the host PM. Therefore, the cell wall and apoplastic space represent the 'front line' for active defense against microbial pathogens. The primary defense against invasion of fungal and oomycete pathogens is penetration resistance, the central features of which are a localized reinforcement of the cell wall (papilla formation) associated with callose deposition, localized accumulation of antimicrobial secondary metabolites (phytoalexins) and secretion of a suite of defense

proteins (Hammerschmidt, 1999; Dixon, 2001; Maor and Shirasu, 2005; van Loon *et al.*, 2006; Hardham *et al.*, 2007). In addition, microscopic observations show that various membranous subcellular structures, for instance H_2O_2-containing vesicles and the so-called multivesicular bodies, accumulate at infection sites, possibly to release their cargos of antimicrobial compounds (Hückelhoven *et al.*, 1999; An *et al.*, 2006). These responses are dependent on secretion/exocytosis and vesicle transport.

A report by Bais *et al.* (2005) provides indirect evidence for the role of secretion in nonhost resistance in *Arabidopsis*–bacteria interactions. The authors show that tissue-specific resistance to a wide range of bacterial pathogens is dependent on the exudation of root-derived antimicrobial metabolites, and that *Pseudomonas syringae* pv. *tomato* is capable of blocking their synthesis and/or secretion to infect the roots and cause disease in a type III secretion-dependent manner (Bais *et al.*, 2005). Another important finding on the activation of the secretory system in response to pathogens came from an extensive survey of genes upregulated by NPR1 in an inducible system (Wang *et al.*, 2005). Along with the expected *PR* genes (*PR-1*, *PR-5*), the expression profile included various genes encoding protein folding and secretory machinery components such as *BiP2*, *DAD1* and *SEC61a*. Indeed, following treatment with an SA analog, null mutants in these genes resulted in compromised secretion of *PR-1* and enhanced susceptibility to *P. syringae* pv. *maculicola* compared to wild type. The NPR1-responsive regulation of secretion-related genes is through *TL1*, a novel cis element distinct from and independent of the TGA-binding *as-1* element (Wang *et al.*, 2005). These data suggest that preparation of the cell for enhanced secretion is controlled by different, unknown NPR1-dependent transcription factors (TFs). Similarly, in animals, B-cells boost the capacity of their secretion machinery before antibody secretion (van Anken *et al.*, 2003).

Exocytosis is dependent on vesicle transport, docking and membrane fusion at the PM. In all eukaryotes, the fusion of membrane-coated vesicles with lipid bilayer target membranes is mediated by a specialized class of proteins, the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). While plants and animals share mechanisms for vesicle formation and fusion, plant cells exhibit a more complex endomembrane system than animal cells, with distinct storage and lytic compartments and different vesicle trafficking routes (Jürgens, 2004). Consequently, about 60 genes in *Arabidopsis* encode SNAREs, compared to 24 in yeast and at least 35 in humans (Sanderfoot, 2007). SNAREs can be classified based on their subcellular localization and function or by structural differences in the SNARE motif; functional classification divides SNAREs into either target-membrane or vesicle-associated SNAREs (t- or v-SNAREs, respectively), while the structural classification is based on the occurrence of either a conserved glutamine or arginine residue in the center of the SNARE domain (Q- or R-SNAREs, respectively) (Lipka *et al.*, 2007). t-SNAREs are further subdivided into proteins that contain one SNARE motif, a C-terminal transmembrane (TM)

domain and diverse N-terminal domains, or smaller proteins that contain two SNARE motifs connected by a flexible linker, and no TM domain. The former class of t-SNAREs is frequently referred to as syntaxins, whereas the latter is defined as synaptosome-associated protein 25 (SNAP-25)-like proteins in *Arabidopsis* based on similarity to their mammalian counterparts. Likewise, vesicle resident v-SNAREs are often described as VAMPs (vesicle-associated membrane proteins). For the purposes of this chapter, we refer to SNAREs broadly as syntaxins, SNAP-25-like proteins and VAMPs. The membrane fusion process is driven by conformational and free-energy changes associated with intermolecular interactions between VAMPs and syntaxins; a typical SNARE complex is composed of a four-helix bundle of intertwined SNARE domains contributed by one VAMP located on the vesicle, and one each of a syntaxin and SNAP-25-like protein on the target membrane (Jahn and Scheller, 2006; Lipka *et al.*, 2007). In addition, syntaxins exhibit an N-terminal autoinhibitory domain composed of three helices that can interact with the SNARE domain, preventing interaction with other SNAREs, suggesting that SNARE-mediated vesicle fusion events are tightly regulated (Fernandez *et al.*, 1998; Lipka *et al.*, 2007). SNAREs have recently emerged at the forefront of molecular and genetic research into the multilayered immune system of plants.

A breakthrough study that focused attention on vesicle-mediated secretion and plant immunity came from the genetic dissection of nonhost resistance using the grass powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*), a barley pathogen that does not normally colonize *Arabidopsis*. The majority of penetration attempts by this pathogen result in failure at the cell wall and absence of intracellular fungal structures. In *pen* (*penetration*) mutants, *Bgh* is able to overcome this preinvasive immunity, resulting in enhanced penetration (Collins *et al.*, 2003). Further growth of haustoria and hyphae are subsequently halted after penetration, suggesting that *pen* mutants do not affect postinvasive immunity. The activation of postinvasive defenses after successful *Bgh* penetration resulted in two phenotypes detectable by fluorescence microscopy: autofluorescence resulting from single-cell death and aniline blue epifluorescence staining of callose deposition (Collins *et al.*, 2003). Because postinvasive responses are not highly activated in the absence of penetration in a wild-type background, these phenotypes were utilized in two separate screens for *pen* mutants. At least four *PEN* genes were discovered. *PEN1* (also called *SYNTAXIN OF PLANTS 121* [*AtSYP121*]) encodes a PM-resident syntaxin, indicating that the secretory machinery is essential for preinvasive defense (Collins *et al.*, 2003). *PEN2* and *PEN3* (also called *PLEIOTROPIC DRUG RESISTANCE 8* [*PDR8*]) encode a glycosyl hydrolase and an ABC transporter, respectively (Lipka *et al.*, 2005; Stein *et al.*, 2006). Another gene, *PEN4*, has recently been cloned and awaits further characterization (M. Lim and S. Somerville, personal communication).

A detailed examination of the subcellular localization of GFP-PEN1 by Assaad *et al.* (2004) showed that fluorescence, localized to the PM in uninduced

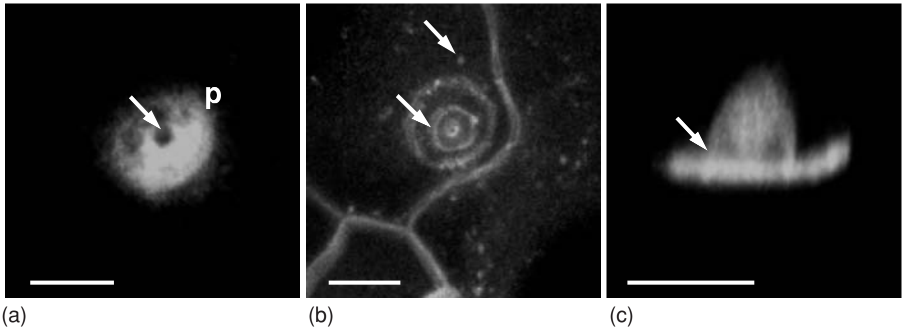

Figure 7.2 Accumulation of GFP-PEN1 at *Arabidopsis* – powdery mildew infection sites. Figures show three-dimensional reconstructions of image stacks. Images were taken 17–24 h after inoculation with *Bgh*. (a) Accumulation of GFP-PEN1 inside a papilla. Arrow points to the penetration hole, in which no GFP-PEN1 is seen. (b) Concentric rings of GFP-PEN1 fluorescence form a 'bull's eye' at the penetration hole. Arrow points to a 'vesicle' ~1 μm in diameter. (c) The second concentric ring of the bull's eye (arrowheads in b and c) is the rim of a hollow cup-shaped structure. p, papilla. Scale bar, 10 μm. Reproduced with permission from Assaad *et al.* (2004), copyrighted by the American Society for Cell Biology.

cells, accumulated at papillae upon infection with both host and nonhost powdery mildews (Fig. 7.2a). Further, papillar localization was seen in concentric rings of GFP-PEN1 fluorescence forming a 'bull's eye' at the penetration hole creating a three-dimensional cup-shaped PM domain surrounding the papillae, and also in the interior of the papillae itself (Figs. 7.2b and 7.2c). GFP-PEN1 is not found exclusively on the PM but also on an ~1-μM-diameter endomembrane compartment seen to form a cloud associated with papillae (Fig. 7.2b). The onset of papillae formation was significantly delayed in the *pen1-1* mutant. Normally, *Bgh* penetration as well as GFP-PEN1 accumulation typically occurs around 12 hours after inoculation – in *pen1-1* the delay in papilla formation is about 2 hours, suggesting that this delay causes the decreased penetration resistance observed in the mutants (Assaad *et al.*, 2004). Since papillae formation is delayed but not abolished, it is likely that there is redundancy at the level of PM-resident syntaxins.

The barley ortholog of *PEN1*, *ROR2* (*REQUIRED FOR mlo RESISTANCE 2*), is implicated in penetration resistance in *mlo* (*mildew resistance locus o*) mutants (Collins *et al.*, 2003). In barley, successful penetration of the cell wall by *Bgh* is dependent on the presence of the plant-specific seven-transmembrane MLO protein (Panstruga, 2005). Barley genotypes lacking functional MLO are resistant against all known isolates of *Bgh*; loss-of-function alleles of *ROR2* partially compromise this resistance, suggesting that MLO might directly or indirectly antagonize ROR2 function (Freialdenhoven *et al.*, 1996). Additionally, MLO and ROR2 have been shown to interact directly *in planta* and, in response to fungal challenge, these proteins accumulate in stable,

disk-shaped patches of PM at the sites of attack (Collins *et al.*, 2003; Bhat *et al.*, 2005). Overexpression of MLO leads to enhanced susceptibility to powdery mildew, rendering all epidermal cells susceptible to the invading fungus (Kim *et al.*, 2002). This is consistent with a negative regulatory role for MLO in penetration resistance. As discussed, the papilla response is delayed in *pen1* mutants, which is the converse of the *mlo* phenotype in barley, where papillae formation occurs earlier than in wild type (Assaad *et al.*, 2004). Indeed, absence of MLO is associated with the spontaneous deposition of callose and papillae formation in unchallenged plants (Panstruga, 2005). Furthermore, *Arabidopsis PEN1* coding sequence driven by the barley *ROR2* promoter complemented the penetration phenotype in *ror2-1* mutant plants (Collins *et al.*, 2003), suggesting that PEN1 and ROR2 are functionally homologous syntaxin family members. Another gene required for full *mlo* resistance, *HvSNAP34*, encodes a SNAP-25-like protein that interacts directly with the syntaxin ROR2 and may represent a binary SNARE complex at the PM (Collins *et al.*, 2003; Schulze-Lefert, 2004). Silencing of this gene resulted in suppression of the *mlo* resistance phenotype, similar to that of *ror2* mutants.

Does *mlo*-based resistance also occur in *Arabidopsis*? There are 15 genes encoding homologs of barley *MLO* in the *Arabidopsis* genome (Chen *et al.*, 2006). Similar to the *mlo* mutant of barley, defects in *Arabidopsis* co-orthologs of *MLO* result in spontaneous callose deposition, ROS production and leaf chlorosis (Consonni *et al.*, 2006). A T-DNA insertion in one of these, *AtMLO2*, results in partial resistance to the virulent powdery mildew species *Golovinomyces orontii*, whereas the triple mutant *Atmlo2 Atmlo6 Atmlo12* (representing a phylogenetic clade of three *MLO* co-orthologs) is completely resistant to *G. orontii* and *G. cichoracearum*, another powdery mildew species (Consonni *et al.*, 2006). Initial data show that AtMLO2 exhibits focal localization at attempted powdery mildew entry sites (R. Panstruga, personal communication). Since *mlo*-mediated resistance in barley requires the syntaxin ROR2, it was predicted that its ortholog PEN1 may be required for *Atmlo2* resistance in *Arabidopsis*. Indeed, the *Atmlo2 pen1* double mutant exhibits near wild-type levels of *G. cichoracearum* penetration rates, although no significant increase in conidiophore production (Consonni *et al.*, 2006). Moreover, it was recently shown that mutations in *PEN2* or *PEN3* (discussed below) restored pathogen entry and significantly enhanced conidiophore formation in the *Atmlo2 pen2* and *Atmlo2 pen3* double mutants, indicating that PEN2 and PEN3 may have additional roles in postinvasion host defense.

While a mechanism for the activity of MLO in exocytosis remains elusive, MLO interacts with calmodulin – the strength of this Ca^{2+}-dependent interaction increases transiently during fungal pathogenesis (Kim *et al.*, 2002; Panstruga, 2005). Also, loss of calmodulin binding diminishes the ability of MLO to negatively regulate defense against powdery mildew in vivo, suggesting that MLO might act as a sensor for cytosolic Ca^{2+}. A pathogen-triggered rapid increase in free Ca^{2+} levels is a common response in plant cells, and there is evidence that these Ca^{2+} fluxes have a role in triggering

resistance (Grant *et al.*, 2000; Ali *et al.*, 2007). In this context, it is interesting that *mlo* exhibits enhanced susceptibility to a range of pathogens unrelated to powdery mildew (Panstruga, 2005). ROR2 has been shown to interact directly with MLO in living barley leaf epidermal cells by fluorescence resonance energy transfer; likewise, PEN1 interacts with AtMLO2 in the yeast split ubiquitin two-hybrid-like assay (R. Panstruga, personal communication). Therefore, it is likely that powdery mildew pathogens have evolved to negatively modulate syntaxin-dependent vesicle fusion at the PM by corrupting MLO, which could sequester ROR2 in an inactive complex (Schulze-Lefert, 2004; Panstruga, 2005).

As described, the fusion of vesicles requires three types of SNARE proteins: a VAMP located on the vesicle, and one each of a syntaxin and SNAP-25-like protein on the target membrane. So far, PEN1/ROR2 syntaxins (and others, see below) and HvSNAP34, a SNAP-25-like protein, have been described in defense responses; involvement of VAMPs is so far not documented. The first evidence for a ternary plant SNARE complex involving all three classes of SNARE proteins is provided by the identification of members of the *At*VAMP72 family associated in a complex with PEN1 and *At*SNAP33, the *Arabidopsis* ortholog of HvSNAP34 (C. Kwon and P. Schulze-Lefert, personal communication). It will be interesting to test whether defects in *AtSNAP33* or *At*VAMP72 affect penetration resistance.

While most evidence points to a positive role of PEN1 in penetration resistance, a recent study reveals that this syntaxin, and its closest homolog *At*Syp122, can serve as negative regulators of other defense-signaling pathways (Assaad *et al.*, 2004; Zhang *et al.*, 2007). The authors conclude that SA, jasmonic acid (JA) and ethylene defense pathways are all activated in *pen1 syp122* double mutants, resulting in a necrotic, dwarfed phenotype and enhanced resistance to virulent pathogens (Fig. 7.3). Mutations in the SA-signaling genes *EDS1*, *EDS5*, *SID2*, *NPR1* or introduction of the bacterial salicylate hydroxylase-encoding gene *NahG* (Friedrich *et al.*, 1995) all partially complement the necrosis and dwarfism of the double syntaxin mutant – earlier studies have shown that defects in components of the SA, JA and ET defense pathways do not affect nonhost penetration resistance, with the exception of a slight effect in *eds1* (Zimmerli *et al.*, 2004; Zhang *et al.*, 2007). So far, the mechanism for how PEN1 and SYP122 inhibit these defense-signaling pathways is unclear, although it has been speculated that they may be required for exocytotic processes separate to those PEN1 has in penetration resistance, such as apoplastic scavenging of ROS, or the trafficking of ion channels. Indeed, recent evidence suggests that PEN1 has a role in the distribution of a K^+ channel protein to PM microdomains (Sutter *et al.*, 2006). As the expression of *PEN1* and *SYP122* increases during pathogen attack and the nonfunctional SYP121-3 protein exhibits relatively high constitutive abundance in *syp121-3* plants, it is likely that these represent a negative feedback loop to limit defense responses and syntaxin protein levels in wild-type plants (Assaad *et al.*, 2004; Zhang *et al.*, 2007). Consistent with these data, transcript levels of

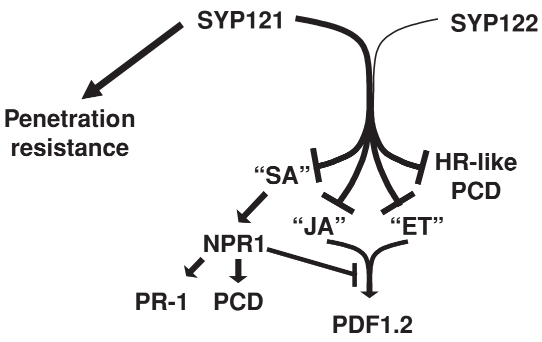

Figure 7.3 A model integrating penetration resistance and several other defense mechanisms through SYP121 and SYP122. SA, JA and ET = salicylic acid-, jasmonic acid- and ethylene-signaling pathways, respectively. PCD, programmed cell death. Reproduced with permission from Zhang *et al.* (2007), published by Blackwell Publishing.

AtSNAP33 (the *Arabidopsis* ortholog of *HvSNAP34*), *PEN3/PDR8* and *PEN2* are responsive to host pathogens or PAMPs (Wick *et al.*, 2003; Zipfel *et al.*, 2004; Kobae *et al.*, 2006; Stein *et al.*, 2006). It is yet unknown whether *At*SNAP33 interacts with PEN1 or has a role in penetration resistance or defense in *Arabidopsis*.

Another strategy employed to find defense-related components of the exocytosis/secretion machinery assayed for proteins phosphorylated at the PM in response to pathogen attack or PAMPs. Two syntaxins, *At*Syp132 and the aforementioned *At*Syp122, were found in a proteomic survey of PM-residing polypeptides that became phosphorylated upon flg22 elicitation in suspension-cultured *Arabidopsis* cells (Nühse *et al.*, 2003; Kalde *et al.*, 2007). Additionally, the ABC transporter PEN3 was found to be a target of flagellin-induced phosphorylation (Nühse *et al.*, 2004). In the case of Syp132, Kalde *et al.* (2007) investigated whether this syntaxin plays a role in defense against bacterial pathogens, but were unable to isolate mutants in *Arabidopsis*. Instead, they employed transient virus-induced gene silencing (VIGS) in *Nicotiana benthamiana* to examine the biological function of the putative ortholog. *N. benthamiana* expressing the tomato *R* gene *Pto* is resistant to *P. syringae* pv. *tabaci* expressing *AvrPto* in a gene-for-gene dependent manner; silencing of *NbSyp132* compromised *Pto*-mediated resistance, resulting in enhanced bacterial growth and manifestation of disease symptoms (Kalde *et al.*, 2007). Silencing *NbSYP121*, the *N. benthamiana* ortholog of *PEN1*, had no effect on Pto-mediated resistance to AvrPto, indicating that the effect was specific for silencing *NbSYP132*. While *Nb*SYP132 was found to be required for secretion of PR-1 and this correlated with SA-dependent resistance to virulent bacteria, silencing of *NbSyp132* did not suppress PAMP-mediated responses or *Pto*-dependent cell death (Kalde *et al.*, 2007). These data suggest that *Nb*Syp121 and *Nb*Syp132 contribute to different branches of defense

and imply that PR-1, along with other defense proteins or compounds, requires *Nb*SYP132 for proper secretion to evoke resistance against the invading pathogen. Moreover, the GFP-tagged barley homolog of SYP132 accumulated beneath appressoria at attempted invasion sites in barley epidermal cells in response to pathogen elicitation (Bhat *et al.*, 2005).

In a separate study by Heese *et al.* (2005), the proposed *Nicotania tabacum* ortholog of PEN1 and HvROR2, *Nt*SYP121 (Leyman *et al.*, 1999), was phosphorylated rapidly and transiently in transgenic tobacco expressing the tomato *R* gene *Cf-9* in response to its cognate elicitor Avr9, while flg22 had no effect. Furthermore, transcript levels of *NtSYP121* were elevated by the Avr9 elicitor but not by flg22 (Heese *et al.*, 2005). These two studies, together with the contrasting roles of *At*SYP121 and *At*SYP122 as negative regulators of SA-, JA- and ET-signaling pathways highlighted by Zhang *et al.* (2007), suggest that syntaxins are involved at multiple levels of plant defense.

Besides *PEN1*, to date two other genes were reported from the screen for enhanced penetration of *Bgh* in *Arabidopsis*: *PEN3/PDR8* and *PEN2*. Like PEN1, the ABC transporter PEN3/PDR8 localizes to the PM in uninfected cells but concentrates at penetration sites when challenged with fungal pathogens (Stein *et al.*, 2006). PEN3/PDR8 likely has a role in pre- and postinvasion resistance since infection of the *pen3* mutant with barley powdery mildew resulted in enhanced penetration and increased secondary hyphae growth compared to wild type, suggesting a compromised restriction of haustorium function after penetration. Besides *Bgh*, *pen3* exhibited enhanced susceptibility to two other biotrophic pathogens that *Arabidopsis* is a nonhost to pea powdery mildew fungus (*Erysiphe pisi*) and the potato late blight oomycete (*Phytophthora infestans*) (Stein *et al.*, 2006). It is proposed that PEN3/PDR8 exports toxic secondary metabolites or antimicrobial peptides to the apoplast at sites of attempted invasion (see Chapter 8). While the substrate of PEN3/PDR8 remains unknown, members of the PDR subfamily of ATP transporters in tobacco are involved in secretion of diterpene sclareol, an antifungal compound, and are thus implicated in pathogen response in tobacco (Jasinski *et al.*, 2001; Stukkens *et al.*, 2005). Interestingly, *pen3* was more resistant to the normally virulent *G. cichoracearum* – this enhanced resistance was coincident with chlorosis and a hyperactivated SA-signaling pathway (Stein *et al.*, 2006). Consistent with this, knockouts of *PEN3/PDR8* result in constitutive activation of *PR* genes, HR-like cell death and enhanced resistance to virulent *P. syringae* pv. *tomato* bacteria (Kobae *et al.*, 2006).

PEN2, a glycosyl hydrolase, is localized to peroxisomes that accumulate to high levels at attempted infection sites (Koh *et al.*, 2005; Lipka *et al.*, 2005). It is likely that PEN1 and PEN2 represent components of two distinct entry resistance mechanisms, based on two lines of evidence: the cumulative effect of *pen1* and *pen2* null mutants on *Bgh* invasiveness, and the observation that PEN2 exhibits a broader spectrum of biological activity than PEN1. As in *pen3* mutants, loss of *PEN2* results in enhanced susceptibility to the inappropriate pathogens *Bgh*, *E. pisi* and *P. infestans* (Lipka *et al.*, 2005). The

functions of PEN2 and PEN3, on the other hand, may be interdependent since *pen2* partially suppresses hyperactive cell death in *Arabidopsis* powdery mildew (*G. cichoracearum*)-infected *pen3* mutants (Stein *et al.*, 2006). Additionally, expression profiling showed that SA pathway-associated genes and SA-dependent downstream markers are upregulated in *pen3* mutants; this set includes PAD4, SID2, EDS1, EDS5, certain PR proteins, glucanases and chitinases (Shah, 2003). These data led Stein *et al.* (2006) to propose a model whereby PEN2, along with other enzymes, may activate related toxins by hydrolysis of nontoxic precursor metabolites – the toxins are subsequently exported by PEN3 to the apoplast at sites of attempted fungal invasion. PEN2-activated toxins might thus accumulate intracellularly in response to *G. cichoracearum* in *pen3* mutants, thereby spuriously activating the SA pathway and cell death.

It is thought that the actin cytoskeleton plays a pivotal role in polarized responses to pathogens by providing tracks for organelle and vesicle traffic to the infection site (Hardham *et al.*, 2007). For example, polarized transport of PEN2-containing peroxisomes to papillae is dependent on actin (Mano *et al.*, 2002; Koh *et al.*, 2005). In vivo monitoring of GFP-tagged organelle-specific reporters shows that pathogen perception induces the formation of extensive actin bundles radiating from penetration sites and the redistribution of ER and Golgi production toward the infection zone, likely for activation of increased secretion (Takemoto *et al.*, 2003). Further evidence comes from pharmacological and genetic studies; depolymerization of actin compromised nonhost resistance to *Colletotrichum truncatum* fungi in *Arabidopsis*, and ectopic expression of *actin depolymerizing factor-encoding gene* (*ADF*) resulted in a breakdown of basal resistance to virulent *Bgh*- and *mlo*-mediated resistance in barley (Shimada *et al.*, 2006; Miklis *et al.*, 2007). However, work by Bhat *et al.* (2005) showed that targeted accumulation of MLO and ROR2 at attempted penetration sites was not affected by overexpression of *ADF* in barley, raising the question of how MLO-syntaxin microdomains are formed. Some excellent reviews highlight numerous descriptive and pharmacological inhibitor studies that further demonstrate the importance of cytoskeletal rearrangements in the plant cells response to pathogens (Schmelzer, 2002; Takemoto and Hardham, 2004).

Since vesicle trafficking and extracellular secretion are clearly important for several aspects of plant pathogen defense, it is predicted that virulent pathogens would target these processes through virulence effector proteins. This was recently shown to be the case in a study by Nomura *et al.* (2006). In a search for host cell targets, *Arabidopsis* MIN7 was identified as an interactor of the *P. syringae* effector protein HopM1. *AtMIN7* encodes one of the eight members of the *Arabidopsis* adenosine diphosphate (ADP) ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) protein family, key components of the vesicle trafficking system in eukaryotic cells (Mossessova *et al.*, 2003). After infection by virulence-compromised bacteria lacking *HopM1*, knockout mutations in *AtMIN7*, but not in other ARF/GEF-encoding

genes, exhibit increased susceptibility and a reduced callose deposition response compared to wild-type plants. Evidence points to a HopM1-triggered ubiquitination and proteasomal degradation of *At*MIN7, resulting in the elimination of a component of the vesicle trafficking pathway and suppressing extracellular cell wall-associated host defense (Nomura *et al.*, 2006). Other bacterial effectors have been causally linked to the suppression of papillae-associated extracellular responses (Grant *et al.*, 2006). One of these, AvrPto, inhibits callose deposition during infection and interacts with proteins implicated in vesicular trafficking (Bogdanove and Martin, 2000; Hauck *et al.*, 2003; Zheng *et al.*, 2005).

7.2.1 Behind enemy lines: endocytosis and PAMP-triggered resistance

As mentioned in the Introduction, perception of PAMPs is mediated by cognate pathogen recognition receptors. In animals, one family of these are the Toll-like receptors (TLRs) that initiate inflammatory responses in response to PAMPs and represent the front line of innate immune responses (Akira *et al.*, 2006). One such protein, TLR4, is the receptor for lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria. LPS perception triggers internalization of TLR4 into endosomes and enhances its subsequent ubiquitination, suggesting receptor degradation (Husebye *et al.*, 2006). TLR4 contains three tyrosine-based tetrapeptide motifs (YxxΦ), a known endocytotic-sorting signature that mediates vesicle budding (Honing *et al.*, 2005).

While well-studied in animals, surface receptor endocytosis is a new research field in plant cell biology in the study of plant immune responses (Robatzek, 2007). Recent data suggest that this innate immune response is shared between kingdoms. For example, endocytosis of *Xanthomonas campestris* LPS occurs in nonhost tobacco, in a manner probably similar to that of TLR4/LPS in animals, although a cognate receptor is not yet known (Gross *et al.*, 2005). Also, the fungal PAMP ethylene-inducing xylanase (EIX) induces *PR* expression and HR in specific cultivars of tomato and tobacco. The tomato transmembrane receptor *Le*Eix2, which binds to EIX and mediates downstream responses, contains the conserved endocytosis signal YxxΦ in its cytoplasmic tail; mutation of this putative signal abolishes EIX-dependent HR (Ron and Avni, 2004). Similarly, the receptor mediating recognition of the bacterial elongation factor Tu (EF-Tu) and the tomato R proteins Ve, Cf4 and Cf9 all possess the endocytosis signature at their C-terminus, suggesting a conserved role of receptor endocytosis in PAMP- and R-protein-mediated responses (Kawchuk *et al.*, 2001; Zipfel *et al.*, 2006; Robatzek, 2007).

The first direct evidence of endocytosis of a plant immune receptor comes from a landmark study by Robatzek *et al.* (2006), in which the PM-resident receptor-like kinase FLS2 was shown to be internalized in response to stimulation by its ligand, flg22. Minutes after elicitation, FLS2-GFP fluorescence

disappeared from the PM and accumulated in intracellular vesicles – these vesicles are likely trafficked for degradation, since prolonged treatment resulted in a complete loss of GFP fluorescence (Robatzek *et al.*, 2006). Upon withdrawal of flg22, PM-residing FLS2-GFP was replenished in a cyclohexamide-sensitive manner, indicating that FLS2 protein is synthesized *de novo* and not recycled. Neither an inactive variant of flg22 from *Agrobacterium tumefaciens* nor elicitation by another PAMP recognized by a different receptor results in internalization of FLS2, suggesting that endocytosis of this PRR is specific to flg22 stimulation and is not a result of cellular changes induced by general PAMPs. A variant FLS2 mutated in Thr 867, a potential phosphorylation site, binds flg22 normally, but is impaired in flg22 responses and endocytosis. Furthermore, as in TLR4, FLS2 contains a ubiquitination motif that, when mutated, compromises receptor endocytosis and downstream signaling but not early responses such as flg22-triggered ROS production (Robatzek *et al.*, 2006). Therefore, the authors suggest that FLS2-mediated flg22 signaling is initiated at the PM and continues from intracellular compartments where it is subjected to degradation (Robatzek, 2007). Inhibition of flg22-triggered endocytosis of FLS2 by the small molecule triclosan, and the identification of a subunit of the fatty acid synthase type II complex as one likely target of triclosan, suggests a role for signaling lipids in this process (Serrano *et al.*, 2007).

The aforementioned processes, outlined in Fig. 7.4, highlight the cell periphery as the site of pathogen perception and initiation of defense responses. Recent studies reveal multiple aspects of nucleocytoplasmic trafficking that link pathogen perception and cytoplasmic signal transduction to defense gene expression.

7.3 At headquarters – nucleocytoplasmic trafficking in plant immunity

Nucleocytoplasmic trafficking is a fundamental process in all eukaryotes that mediate the bidirectional import and export of all proteins and RNA through the nuclear pore complex (NPC). The NPC is a large macromolecular structure that spans the double membrane of the nuclear envelope, and is composed of nucleoporins (Nups) that localize to protein subcomplexes within the greater pore complex. While some Nups are structural and required for NPC assembly, studies show that certain Nups are mobile and not permanently associated with the NPC (Tran and Wente, 2006). Nups lining the interior of the nuclear pore exhibit phenylalanine-glycine (FG) repeats that are thought to provide transient, low affinity-binding sites for transport receptors (Frey *et al.*, 2006). These receptors, collectively termed karyopherins (Kaps), recognize exposed nuclear localization signals (NLSs) and nuclear export signals (NESs) to mediate the bidirectional nucleocytoplasmic shuttling

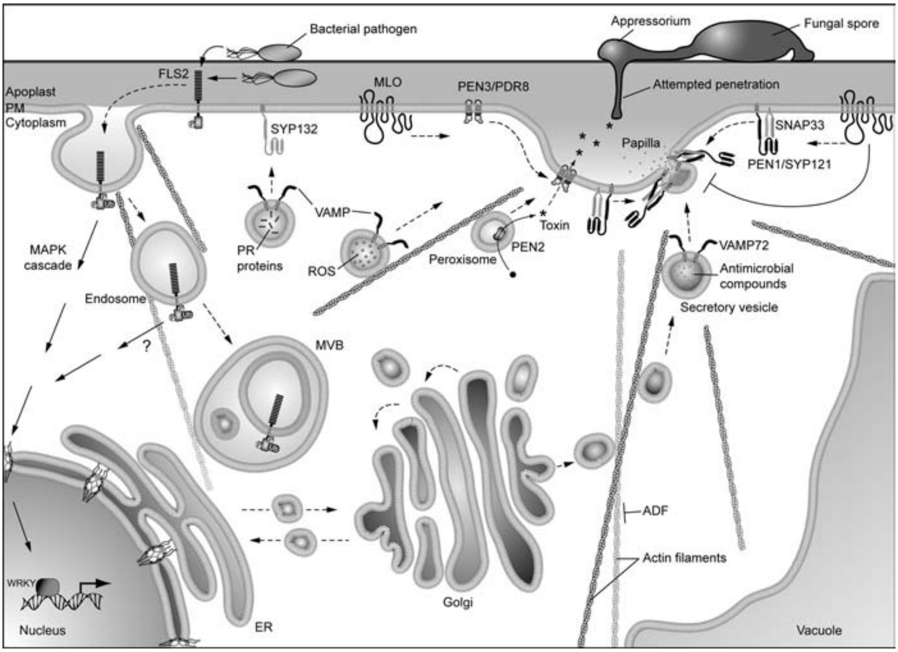

Figure 7.4 Conceptual summary of microbe-triggered dynamic cellular responses in plants. On the left side flg22-mediated internalization of the pattern recognition receptor FLS2 and its subsequent accumulation via endosomes in multivesicular bodies (MVBs) is shown. On the right side, cellular responses to attempted fungal ingress are shown; these involve actin filament-mediated focal transport of vesicles to and SNARE protein-dependent fusion of vesicles with the plasma membrane (PM), a process that is thought to be negatively regulated by MLO. Vesicle trafficking pathways are embedded into the cellular membrane trafficking system linking the PM with the endoplasmic reticulum (ER), Golgi and vacuoles. Dashed arrows indicate vesicle movement and protein re-localizations. ROS, reactive oxygen species; ADF, actin depolymerizing factor; P, phosphorylation; Ub, ubiquitination. For further details, see text.

of these cargo proteins. While Nups are well characterized in humans and yeast, relatively little is known in plants. For an overview on nuclear transport in plant cells and the composition of the plant NPC, the reader is referred to comprehensive reviews by Merkle (2001, 2003) and Meier (2007).

The relationship between plant pathogen defense and nucleocytoplasmic trafficking has been intensively studied in recent years. The nucleus is the site for all transcription, and therefore transcription factors (TFs) involved in regulating pathogen-responsive genes must be imported at some point. It is the rapid and dynamic regulation of these TFs in response to pathogen attack, in many cases by interacting signal transducers or phosphorylation cascades that signal from the cytoplasm to the nucleus, which determines the efficacy of the host response. Nucleocytoplasmic partitioning is a well-studied feature of innate immunity in animals, a process that largely depends upon

the induced translocation of NF-κB from the cytoplasm to the nucleus. In uninduced cells, NF-κB proteins are predominantly cytoplasmic and interact with members of the inhibitory IκB family (Ghosh *et al.*, 1998). Depending on the type of IκB, these proteins either mask NF-κB's classic NLS, sequestering it in the cytoplasm, or prevent high steady-state accumulation of these complexes in the nucleus through an NES in IκB (Huang *et al.*, 2000; Malek *et al.*, 2001). Upon induction, IκB is degraded, and activated NF-κB accumulates in the nucleus, stimulating the expression of immune-response genes. MAPK-signaling cascades terminating in the nucleus are another essential feature shared by animals and plants, fulfilling important regulatory functions that modulate defense against pathogens (Tena *et al.*, 2001; Nürnberger *et al.*, 2004). Activated MAPKs correlate with the upregulation of many *PR* genes in several experimental systems, suggesting that they play an important role in gene transcription (Pedley and Martin, 2005). MAPKs have been shown to translocate to the nucleus in rice (Cheong *et al.*, 2003) and in cultured parsley cells in response to Pep13, a peptide elicitor derived from the oomycete pathogen *Phytophthora* (Lee *et al.*, 2004).

Several recent studies point to nucleocytoplasmic trafficking as a key regulatory step of effector-mediated and basal resistance. Components of the nuclear transport machinery were found in genetic screens for regulators of R-protein-mediated resistance and as interactors of R proteins. Key regulators of plant defense are reported to be regulated across the nuclear envelope in a spatio-temporal manner reminiscent of NF-κB. Notably, intracellular R proteins themselves have been found to function in the nucleus. Two studies of paramount interest showed that NB-LRR R proteins from barley (MLA, CC-type) and tobacco (N, TIR-type) have activities in the nucleus (Burch-Smith *et al.*, 2007; Shen *et al.*, 2007). Nuclear localization was not anticipated since both lack a discernable NLS – the prevailing hypothesis until now was that R proteins, being intracellular immune receptors, were cytoplasmic or associated with the PM to recognize pathogen effectors directly or indirectly (Belkhadir *et al.*, 2004; Jones and Dangl, 2006). However, the recent results fit with accumulating evidence that some pathogen effector proteins are directed to the nucleus and that defects in nuclear trafficking machinery affect R-protein-mediated resistance (see below). Another topical study revealed that RPS4, an *Arabidopsis* TIR-NB-LRR R protein, has nuclear activity conferred by an endogenous bipartite NLS in its C-terminal extension domain (Wirthmueller *et al.*, 2007). With all three R proteins, reducing nuclear accumulation either through addition of an NES sequence or, in the case of RPS4, mutating the endogenous NLS revealed that nuclear localization is essential for effector-mediated resistance.

7.3.1 R-protein activity in the nucleus

Shen *et al.* (2007) analyzed allelic barley CC-NB-LRRs MLA1, MLA6 and MLA10 that confer resistance to powdery mildew *Bgh* expressing the specific

effectors AVR_{A1}, AVR_{A6} and AVR_{A10}, respectively. In vivo imaging of yellow fluorescent protein (YFP)-tagged MLA10 transiently expressed in leaf epidermal cells showed that MLA10-YFP localized to the cytoplasm and nucleus, and likely shuttles between the two compartments. An NES fusion to MLA10-YFP prevents its nuclear accumulation and suppresses defense responses. In stable transgenic barley lines, epitope-tagged MLA1 was also detected in both the nuclear and cytoplasmic fractions, suggesting that the MLA10-YFP localization in the single-cell system reflects the physiological location of some MLA proteins. In addition, a time course revealed an increase in the MLA1 nuclear pool in response to inoculation of *Bgh* carrying its cognate effector AVR_{A1}, but not *Bgh* lacking AVR_{A1}, suggesting that recognition of the effector triggers dynamic nuclear changes in the behavior of the R protein (Shen *et al.*, 2007). The invariant CC domain shared by all three MLAs interacts with two closely related barley WRKY domain-containing TFs, which repress defense responses to *Bgh*. Silencing of *HvWRKY1/2* significantly enhanced resistance to virulent *Bgh*, whereas overexpression of *HvWRKY1/2* resulted in enhanced susceptibility to virulent *Bgh* (Shen *et al.*, 2007). Their overexpression also compromised MLA1-, MLA10- and MLA12-mediated resistance to avirulent *Bgh* isolates expressing the cognate AVR_A effectors. The *Arabidopsis* functional homologs (orthologs) of *Hv*WRKY1/2, *At*WRKY18 and *At*WRKY40 were implicated previously in the repression of basal defense to virulent *Pseudomonas* (Xu *et al.*, 2006) and also exhibited redundant resistance repression activities in the virulent powdery mildew *G. orontii*–*Arabidopsis* interaction (Shen *et al.*, 2007). In barley, transiently expressed MLA10-YFP and cyan fluorescent protein (CFP)-*Hv*WRKY2 fusion proteins co-localized in epidermal cell nuclei; protein association was monitored by Förster resonance energy transfer (FRET) between the YFP and CFP fluorescent tags in the presence and absence of Avr_{A10}. Importantly, using quantitative fluorescence lifetime imaging (FLIM) to detect FRET, the association of MLA10 and *Hv*WRKY2 was dependent on the presence of the cognate effector (Ridout *et al.*, 2006; Shen *et al.*, 2007). A direct nuclear association between MLAs and WRKY transcriptional repressors, induced by the fungal effector, implies a short signaling pathway suited to rapid defense activation. A model for such a mode of receptor-triggered resistance is illustrated in Fig. 7.5a.

Another notable report demonstrates R-protein activity in the nucleus (Burch-Smith *et al.*, 2007). The tobacco TIR type NB-LRR protein N, together with its cognate effector, the p50 helicase domain of the tobacco mosaic virus (TMV) replicase are found in both the cytoplasm and the nucleus. Recognition of p50 by N triggers an HR and confers resistance to TMV (Whitham *et al.*, 1994; Erickson *et al.*, 1999). As with MLA10, fusing an NES to N abolished p50-dependent HR, indicating that N's nuclear localization is indispensable for Avr-triggered defense responses. Using the same strategy, it was shown that the nuclear localization of p50 is not required for HR. These data suggest that N recognizes p50 in the cytoplasm and that the nuclear pool of N has another function (Burch-Smith *et al.*, 2007). Using bimolecular fluorescence

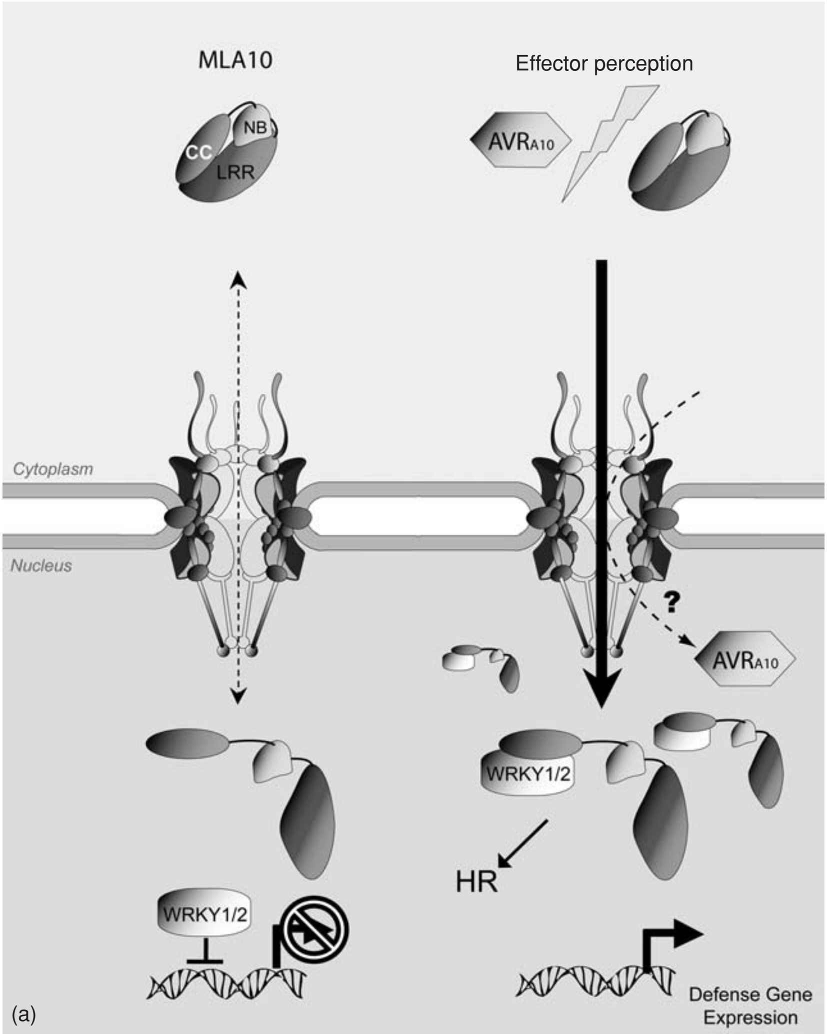

Figure 7.5 A model for nuclear R-protein signaling. The barley MLA10 (CC-NB-LRR) receptor (a) and tobacco N (TIR-NB-LRR) receptor (b) are present in both the cytoplasm and the nucleus. Nuclear localization of either R protein is indispensable for downstream signaling and defense responses. According to the previous model outlined in Wiermer *et al.* (2003), in an un-induced cell some NB-LRR R proteins may continuously cycle between the cytoplasm and the nucleus, and equilibrium is maintained. Upon pathogen effector perception, the nuclear pool of activated R protein increases and, after reaching a threshold concentration, de-represses defense gene expression. (a) The N-terminal CC domain of barley MLA10 interacts with WRKY1/2. It is not known whether Avr_{A10} is also present in the nucleus or where perception occurs. (b) The viral effector p50 is present in both subcellular fractions and is recognized by the N-terminal TIR domain of tobacco N. Host factors may assist this association in vivo. It is unclear whether activated N also binds to WRKY-negative transcriptional regulators. See text for details on these two NB-LRR R proteins. TFs, transcription factors.

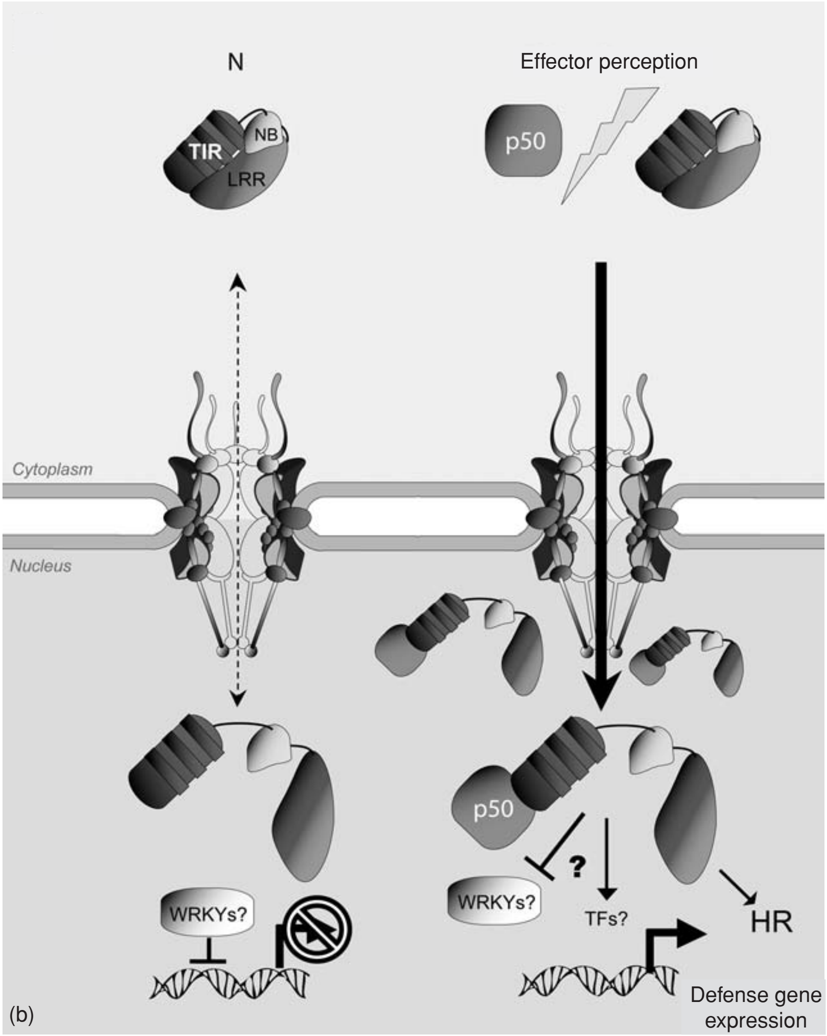

Figure 7.5 (Continued)

complementation (BiFC) imaging in living cells and biochemical assays, this study showed that the TIR domain of N associates with p50, likely in the context of other host factors. This was unexpected since previous evidence pointed to the LRR domain being involved in ligand recognition. Based on this and other studies, a complex, multistep process of N receptor activation by the TMV replicase is emerging, perhaps resulting in activated N shuttling from the cytoplasm to the nucleus subsequent to Avr-dependent activation, as shown in Fig. 7.5b (Burch-Smith *et al.*, 2007; Sheen and He, 2007). Intriguingly, plant-specific transcription factors were previously shown to interact

with the LRR of N – in the context of this study, hinting at a role for R proteins regulating gene transcription (Liu *et al.*, 2004).

A third piece of direct evidence highlighting the nucleus as a key location for the function of intracellular immune receptors is the demonstration that a sub-pool of the *Arabidopsis* TIR-NB-LRR immune receptor, RPS4, that recognizes the bacterial Type III effector AvrRps4, localizes to the nucleus (Wirthmueller *et al.*, 2007). By cellular fractionation and imaging of YFP-tagged fusion proteins, RPS4 was found to distribute constitutively between endomembranes and nuclei. As with MLA and N, a nuclear exclusion strategy was employed to determine whether nuclear localization of RPS4 was required to trigger immune responses. Mutation of the endogenous bipartite NLS in RPS4 abolished *AvrRps4*-triggered resistance and reduced nuclear accumulation in *Arabidopsis*. Transient overexpression of RPS4 in tobacco induces an AvrRps4-independent HR (Zhang *et al.*, 2004). Expression of the *nls* mutant of RPS4 or fusion of an NES to YFP-RPS4 also abolished cell death and resulted in redistribution of YFP-RPS4 from the nucleus to the nonnuclear compartment in *N. benthamiana* leaves. These data indicate that nuclear localization of RPS4 is essential for both an authentic effector-activated and deregulated immune response. Chemically inducible AvrRps4 accumulated in the cytosol suggesting that a spatial separation between RPS4 receptor activation and signaling may exist (Wirthmueller *et al.*, 2007).

Before the identification of MLA10, N and RPS4 as nuclear-targeted R proteins, the only other concrete example of an R protein with activity in the plant nucleus was the atypical RRS1-R of *Arabidopsis*. RRS1-R is unusual in that it contains, along with TIR-NB-LRR domain of other TIR-class R proteins, a C-terminal extension with a putative NLS and a 60-amino acid DNA-binding motif characteristic of the WRKY family of TFs (Deslandes *et al.*, 2002). The presence of WRKY and NLS domains in RRS1-R suggests that this R protein mediates Avr perception and subsequent transcriptional activation of the defense-related transcripts, representing an extremely condensed signaling pathway. It was recently discovered that an in-frame 3-bp insertion mutation in the WRKY domain of RRS1-R that impairs its DNA-binding activity results in constitutive activation of defense responses, suggesting that resistance signaling may be negatively regulated by the WRKY domain of RRS1-R (Noutoshi *et al.*, 2005). One interpretation of these data is that other, typical NB-LRR R proteins might directly activate gene expression by interacting with WRKYs, and that RRS1-R represents an evolutionary 'Rosetta stone' of domain fusion events of functionally related proteins that physically interact (Martin *et al.*, 2003). However, direct interaction between an R protein and WRKY TFs has only been reported for MLA (Shen *et al.*, 2007). In this context, it is interesting that the LRR domain of N was found to interact with plant-specific TFs (Liu *et al.*, 2004).

RRS1-R confers resistance to the bacterial pathogen *Ralstonia solanacearum* expressing PopP2, a member of the YopJ/AvrRxv family of effector proteins that is targeted to the plant cell nucleus. It was suggested that PopP2 may

bind an importin-α via its NLS and that this protein may be a potential virulence target of the avirulence effector that is guarded by RRS1-R (Lahaye, 2004). Nevertheless, RRS1-R and PopP2 interact directly in a yeast-2-hybrid system, and this interaction is essential for inducing accumulation of fluorescent protein-tagged RRS1-R in the nucleus (Deslandes *et al.*, 2003). It remains unclear whether these two proteins first interact in the cytoplasm, shuttling RRS1-R into the nucleus, or if PopP2 interacts with a preexisting nuclear pool of RRS1-R and somehow suppresses its export, allowing R-protein accumulation to levels detectable by fluorescence microscopy. Evidence suggests that other pathogen effector proteins are active in the nucleus, based on conserved NLSs and/or DNA-binding motifs (Zhu *et al.*, 1998; Yang *et al.*, 2000; Szurek *et al.*, 2001; Kemen *et al.*, 2005; Kanneganti *et al.*, 2007). For example, AvrBs3 and AvrBs4, two nearly identical bacterial type III effectors from *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) that are specifically detected by the cognate pepper Bs3 and tomato Bs4 R proteins, respectively, are targeted to the nucleus (Schornack *et al.*, 2006). These Avr proteins exhibit potential C-terminal acidic transcription activation domains (AD) and monopartite NLSs that could possibly hijack the host nuclear transport machinery to gain access to the nucleus; in the case of AvrBs3, the disruption of either the AD or the NLSs affect both *Xcv* virulence and Bs3 recognition (Szurek *et al.*, 2001). The guard hypothesis suggests that Avr-induced modifications of a host target protein trigger activation of matching plant R proteins (Dangl and Jones, 2001). According to this model, the R-protein Bs3 is predicted to be nuclear. However, a very recent report by Römer *et al.* (2007) shows that *Bs3* encodes a structurally novel flavin monooxygenase (FMO) and that AvrBs3 binds and activates the *Bs3* promoter. This suggests a new recognition mechanism in which Avr proteins are detected via their transcriptional activation of cognate *R* genes (Römer *et al.*, 2007). In contrast, the *Bs4*-dependent avirulence activity of AvrBs4 is compromised by deletion of neither its NLS nor AD domains, suggesting that Bs4 recognizes and is activated by AvrBs4 during its transit through the cytoplasm (Bonas *et al.*, 1993; Schornack *et al.*, 2006). While this result points to a separation of virulence and avirulence activity, contrary to predictions based on the guard model, the effect of the C-terminal truncation on virulence of AvrBs4 and direct localization of Bs4 remains to be tested.

The discovery that NB-LRR R receptors have activities in the nucleus led Shen *et al.* (2007) and Burch-Smith *et al.* (2007) to propose a conceptual model that we extended in a recent review (Wiermer *et al.*, 2007). According to this model, in an uninduced cell some NB-LRR R proteins continuously cycle between the cytoplasm and nucleus and equilibrium is maintained. Upon pathogen effector recognition, the nuclear pool of R proteins increases. After the nuclear R-protein concentration reaches a certain threshold, defense gene expression is induced by, for example, derepression of WRKY TFs. Accumulation of the nuclear pool of R proteins could be accomplished by an increase in the rate of nuclear import or a decrease in the rate of nuclear export. Some R proteins are targeted to the nucleus by an NLS and some

are not. Therefore, effector-mediated activation could expose an obscured NLS via conformational changes or recruit additional interacting proteins to affect nuclear shuttling or retention. This model predicts that defects in the NPC or transport machinery might alter resistance by upsetting the ratio of cytoplasmic- to nuclear-localized R proteins. Furthermore, elevated defense gene expression associated with overexpression of *R* genes and constitutively active R-protein variants (i.e. *snc1*, *ssi4*) may be explained by altered nucleocytoplasmic distribution (Shirano *et al.*, 2002; Zhang *et al.*, 2003). The connection between R-protein nuclear activity and effector perception is unknown and awaits further research. Also, it is likely that the cytoplasmic R-protein pool is involved in other, nontranscriptional downstream signaling events, such as those that lead to the HR.

These nucleocytoplasmic R-protein regulation models highlight the significance of the identities of several positive regulators of an autoimmune mutant, *suppressor of npr1-1, constitutive 1* (*snc1*). *snc1* carries a gain-of-function mutation in a TIR-NB-LRR class *R* gene resulting in activation of downstream defense responses in the absence of pathogens, such as constitutive *PR* gene expression, accumulation of SA, and enhanced resistance to virulent *P. syringae* bacteria and *Hyaloperonospora parasitica* oomycete pathogens without spontaneous cell death (Li *et al.*, 2001; Zhang *et al.*, 2003). The pathogen effector recognized by wild-type SNC1 is unknown. Several genes cloned by our group in an *snc1* suppressor screen, *MOS3*, *MOS7* and *MOS6* (for *MODIFIER OF snc1*), encode components of the plant nucleocytoplasmic trafficking machinery, revealing the importance of this process in deregulated R-protein signaling. Subsequent analysis showed that the products of *MOS* genes are required both for basal resistance to virulent pathogens and for resistance mediated by other *R* genes in addition to *snc1*.

MOS3 is the *Arabidopsis* homolog of vertebrate nucleoporin 96 (Nup96) and *Saccharomyces cerevisae* C-Nup145p, which are components of the conserved Nup107-160 nuclear pore subcomplex (termed the Nup84 complex in yeast) (Zhang and Li, 2005). This complex has a crucial role in nuclear pore assembly, since complete depletion of the Nup107-160 complex leads to nuclei entirely lacking pores (Harel *et al.*, 2003; Walther *et al.*, 2003). This subcomplex, which localizes to both sides of the nuclear pore, has been implicated in both mRNA export and mitotic spindle assembly (Belgareh *et al.*, 2001; Vasu *et al.*, 2001; Orjalo *et al.*, 2006). Faria *et al.* (2006) established a selective role for Nup96 in innate and adaptive immunity from studies of Nup96-depleted mice; upon immunization, mice with low levels of Nup96 show impaired antigen presentation and T-cell proliferation. This and previous experiments showed that Nup96 is regulated by interferons and actively participates in the interferon-dependent induction of major histocompatibility complex (MHC) I and MHC II (Enninga *et al.*, 2002; Faria *et al.*, 2006). While the total levels of *MHC I* and *II* mRNAs in Nup96-depleted and control mice are similar, the ratios of nuclear to cytoplasmic levels of these mRNAs are partially but significantly increased in Nup96-depleted macrophages. This partial nuclear

retention likely contributes to lower expression of these proteins. Taken together, the data suggest an ancient role for Nup96 in immunity, possibly due to its role in nuclear export of specific classes of mRNAs, although it remains to be determined whether the defects in disease resistance observed in *mos3* are specific or a pleiotropic phenotype based on nuclear mRNA retention. *MOS3* was found independently in a genetic screen for suppressors of *auxin-resistant 1* (*sar* mutants) and designated *SAR3* (Parry *et al.*, 2006). Another gene, *SAR1*, encoding the *Arabidopsis* homolog of Nup160, was also identified in this screen. *sar1* single-mutant plants exhibit defects in auxin response and in tolerance to cold stress, *mos3* single-mutant plants are early-flowering, and *sar1 mos3* double mutants present variable phenotypes such as severe developmental defects, seedling lethality and infertility, along with nuclear accumulation of mRNA (Parry *et al.*, 2006). Corroborating data from animal systems, the *sar1 mos3* double mutants might represent a nonfunctional orthologous *Arabidopsis* Nup107-160 subcomplex, and the severe phenotypes may be due to impaired NPC assembly. This suggests that *At*Nup96 itself might have specific functions and is not purely a structural protein. Intriguingly, homologs of two additional members of the Nup107-160 complex, Nup85 and Nup133, are essential for microbial symbiosis in the legume *Lotus japonicus* (Kanamori *et al.*, 2006; Saito *et al.*, 2007).

MOS7 encodes another Nup that has sequence homology to vertebrate Nup88 (Y. Cheng and X. Li, unpublished data). In addition to *snc1*-mediated resistance, *MOS7* is essential for resistance to avirulent pathogens mediated by other R proteins, basal resistance and SAR. Its homologs in *Drosophila*, *members only* (*mbo*), is found on the cytoplasmic side of the NPC and is required selectively for activation of innate immune responses to bacterial infection (Uv *et al.*, 2000). In *Drosophila*, Toll signaling upon bacterial infection leads to degradation of the IκB homolog, Cactus, and nuclear accumulation of the Rel/NFκB TFs Dorsal and Dif. In mutant larvae lacking *Dm*Nup88, Dorsal and Dif fail to accumulate in the nucleus (Uv *et al.*, 2000). While overexpression of Nup88 interferes with nuclear export of a GFP-NES reporter construct, the cytoplasmic accumulation of GFP-NES is accelerated in *mbo* mutants – these and other data suggest that Nup88 is modulates protein retention in the nucleus (Roth *et al.*, 2003; Xylourgidis *et al.*, 2006). A possible mechanism for Nup88's role in innate immunity is to selectively trap endogenous TFs and thereby alter expression of Rel/NFκB target genes upon activation of the defense response. Although *mbo* mutant flies fail to accumulate these Rel proteins in the nucleus, they are not affected in the nuclear translocation of several other, unrelated proteins (Xylourgidis *et al.*, 2006). It will be interesting to test if MOS7 is involved in protein retention of any known plant defense regulators. As expected for components of the NPC, GFP-tagged MOS3 and MOS7 localize exclusively to the nuclear rim (Zhang and Li, 2005; Y. Cheng and X. Li, unpublished data).

MOS6 encodes importin-α3, one of at least eight putative α-importins encoded by the *Arabidopsis* genome. A defect in this importin subunit partially

suppresses the constitutive defense responses in *snc1* and displays enhanced susceptibility to a virulent *H. parasitica* oomycete pathogen (Palma *et al.*, 2005). Importins are a conserved family of Kaps, mobile targeting receptors that mediate the bidirectional trafficking of macromolecules across the nuclear envelope. The best understood function of the α-subunit is as an adaptor that links NLS-containing proteins to importin-β, which mediates translocation through the NPC by its ability to interact with Nups (Goldfarb *et al.*, 2004). The deduced MOS6 protein has the typical architecture of all importin-α proteins (except for *At*Imp-α8): an N-terminal autoinhibitory importin-β-binding domain, armadillo repeats containing NLS-binding pockets, and a C-terminal acidic patch that is implicated in the interaction with a specific export receptor for recycling back to the cytoplasm (Goldfarb *et al.*, 2004). The importin-α gene family has undergone considerable expansion during the course of eukaryotic evolution. Whereas the yeast genome encodes a single importin-α, multicellular eukaryotes contain multiple proteins with deduced importin-α function. The existence of multiple importin-α isoforms in animals and plants raises questions about the specificity and efficiency in transport of different NLS-containing proteins. Due to high sequence similarity, it is probable that some *Arabidopsis* importins are functionally redundant. For example, MOS6 (importin-α3) is almost identical to importin-α6, and these two proteins are closely related phylogenetically (Palma *et al.*, 2005). Since all alleles of mos6 only partially suppress *snc1* phenotypes, it will be interesting to see if defects in both *AtImpα6* and *MOS6* suppress *snc1* more fully.

The significance of having multiple importin-α homologs can be inferred from studies in humans in which at least seven different importin-α proteins have been described (Pemberton and Paschal, 2005). The downregulation by RNAi of two ubiquitously expressed human importins, α3 and α5, specifically inhibits cellular proliferation and import of the nuclear protein Ran guanine nucleotide exchange factor (RCC1) – both defects can be rescued by microinjection of recombinant importin-α3 (Quensel *et al.*, 2004). Therefore, nuclear import of RCC1 appears to be dependent on importin-α3 only in humans. In another human study, importin-α3 and importin-α4 were identified as the main importin-α isoforms mediating TNF-α-stimulated NF-κB translocation into the nucleus (Fagerlund *et al.*, 2005). So far, limited examples of importin specificity have been described in plants. In rice, nuclear import of constitutive photomorphogenic 1 (COP1), a repressor of photomorphogenesis regulated by nucleo-cytoplasmic partitioning, is mediated preferentially by the rice importin-α1b (Jiang *et al.*, 2001). Further evidence of specificity comes from a report by Kanneganti *et al.* (2007), showing that *N. benthamiana* importin-α isoforms have distinct preferences for different NLS-carrying proteins. VIGS of two importins, *NbImpα1* and *NbImpα2*, inhibit the nuclear targeting of fluorescent protein-tagged *P. infestans* candidate effectors Nuk6 and Nuk7. In contrast, nuclear import of *P. infestans* Nuk12 and YFP-SV40NLS (a reporter shown previously to require importin-α for nuclear import) is unaffected in these silenced plants (Kanneganti *et al.*, 2007). This

study demonstrates invivo that importin-α isoforms have distinct preferences for different NLS-carrying proteins. While it is possible that other unknown *N. benthamiana* importin-α genes were targeted by VIGS, it is unlikely that all of these were silenced, as nuclear import of the YFP–SV40NLS fusion was not inhibited in the silenced plants. The most plausible explanation for this result is that the nuclear import of the YFP–SV40NLS fusion depends on *N. benthamiana* importin-α isoforms other than *Nb*IMPα1 and *Nb*IMPα2 and close homologs (Kanneganti *et al.*, 2007). This study shows that VIGS is a viable strategy to dissect the roles of different importin-α proteins in specific trafficking of nuclear-localized defense proteins and plant pathogen effectors. Another strategy in *Arabidopsis*, although tedious, would be analysis of double-, triple- and higher-order mutant lines with T-DNA insertions in *Arabidopsis* importin-α-encoding genes to test effects on nucleocytoplasmic trafficking of candidate immune proteins.

A recent report by Tameling and Baulcombe (2007) implicates nucleocytoplasmic trafficking in R-protein-mediated immunity to a plant virus. Resistance to *Potato virus X* (PVX) is mediated by the potato CC-NB-LRR protein Rx via recognition of the viral coat protein (CP) (Bendahmane *et al.*, 1995). In a targeted proteomics approach, proteins in the Rx-signaling complex were identified by affinity purification and mass spectrometry after transient co-expression of tagged Rx fragments (CC or LRR domains) in *N. benthamiana* leaves. In many independent experiments, the purified Rx-CC fragment copurified with a Ran GTPase-activating protein (RanGAP), whereas the purified Rx-LRR fragment did not (Tameling and Baulcombe, 2007). Further characterization revealed that the Rx-CC-interacting protein was RanGAP2, one of two RanGAPs in *N. benthamiana* that are similar to the two RanGAP proteins found in *Arabidopsis*. Silencing of *RanGAP2* by VIGS suppresses Rx-mediated resistance to PVX but does not affect resistance mediated by other NB-LRR proteins such as N or Pto, suggesting that RanGAP2 is required specifically for Rx function. In an independent study, RanGAP2 interacted with not only Rx but also the CC-domains of the close homologs Gpa2, which confers resistance to the potato cyst nematode, and Rx2 (Sacco *et al.*, 2007). The small GTPase Ran is required for directionality of nucleocytoplasmic trafficking through the NPC. Association of Ran-GTP with Kaps inside the nucleus causes the release of import cargos and binding of export cargos, whereas Ran-GTP hydrolysis in the cytoplasm triggers the dissociation of Ran-GDP-Kap-export cargo complexes. Back in the nucleus, Ran-GDP is converted to Ran-GTP by RCC1 (Merkle, 2001). RanGAP stimulates the GTPase activity of Ran-GTP on the cytoplasmic side of the nuclear envelope. Thus, a resulting Ran-GDP versus Ran-GTP gradient over the NE is established by the spatial sequestering of the Ran accessory proteins to maintain the directionality of nucleocytoplasmic transport (Meier, 2007). For the role of RanGAP2 in Rx-mediated resistance, the authors offer three alternative hypotheses: (1) the PVX CP binds to or somehow indirectly perturbs RanGAP2 function and Rx, as predicted by the prevailing Guard Hypothesis, senses the activity of the

effector and is activated, (2) Rx itself is targeted to the nucleus, and interaction with RanGAP acts as a carrier protein at the NE, or (3) subsequent to activation by CP, Rx interacts with RanGAP to influence the trafficking of resistance-related proteins (Tameling and Baulcombe, 2007). The first hypothesis implies that the CC domain of Rx is involved in effector recognition, against the popular notion that the LRR is more important for this function. Recent data revealing that the TIR domain of N, and not the LRRs, associates with the TMV effector p50 argues against this hypothesis (Burch-Smith *et al.*, 2007). Also, a similar mechanism has been proposed for the HRT-dependent resistance to turnip crinkle virus; the CP effector binds to and blocks the nuclear accumulation of a putative TF (Ren *et al.*, 2005). Evidence for the latter two hypotheses is circumstantial, but a growing body of work continues to shed light on the role of nucleocytoplasmic trafficking in resistance signaling. One supporting piece of data is that Rx has been detected in the nucleus (J. Bakker, personal communication). If RanGAP2 itself functions as the carrier for nuclear Rx, which has no detectable NLS, perhaps it can be shown experimentally that RanGAP2 is also required for nuclear accumulation of MLA10, N and other R proteins lacking obvious NLSs. Alternatively, since RanGAP2 is involved in hydrolysis of Ran-GTP and cytoplasmic dissociation of export cargo, inhibition of RanGAP2 by Rx could alter the Ran-GDP/Ran-GTP gradient and thus suppress nuclear export of R proteins or defense regulators, increasing the relative concentration of their nuclear pool. Further work is required on the mechanism of RanGAPs on nucleocytoplasmic localization of defense proteins. Components of the nucleocytoplasmic trafficking machinery implicated in plant–microbe interactions are illustrated in Fig. 7.6.

7.3.2 Regulation of defense proteins by nucleocytoplasmic partitioning

Besides R proteins, shuttling of downstream regulators across the nuclear envelope is likely to be a key regulatory step in defense. In *Arabidopsis*, TIR-NB-LRR-triggered and basal defense responses are dependent on the lipase-like protein ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and its sequence-related interacting partners PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE ASSOCIATED GENE 101 (SAG101), which together constitute a central regulatory node in diverse immune responses (Aarts *et al.*, 1998; Feys *et al.*, 2001; Feys *et al.*, 2005). While the biochemical activities of EDS1, PAD4 and SAG101, or the signal they transduce are not known, there is strong evidence that they process ROS-derived signals as part of a signal potentiation loop (Rustérucci *et al.*, 2001; Wiermer *et al.*, 2005). Several molecularly distinct EDS1 complexes were identified in healthy leaf tissues providing a handle to explore the role of trafficking on EDS1-dependent defense responses. Without pathogen infection, EDS1 and PAD4 localize to both the cytoplasm and the nucleus whereas SAG101 is exclusively nuclear.

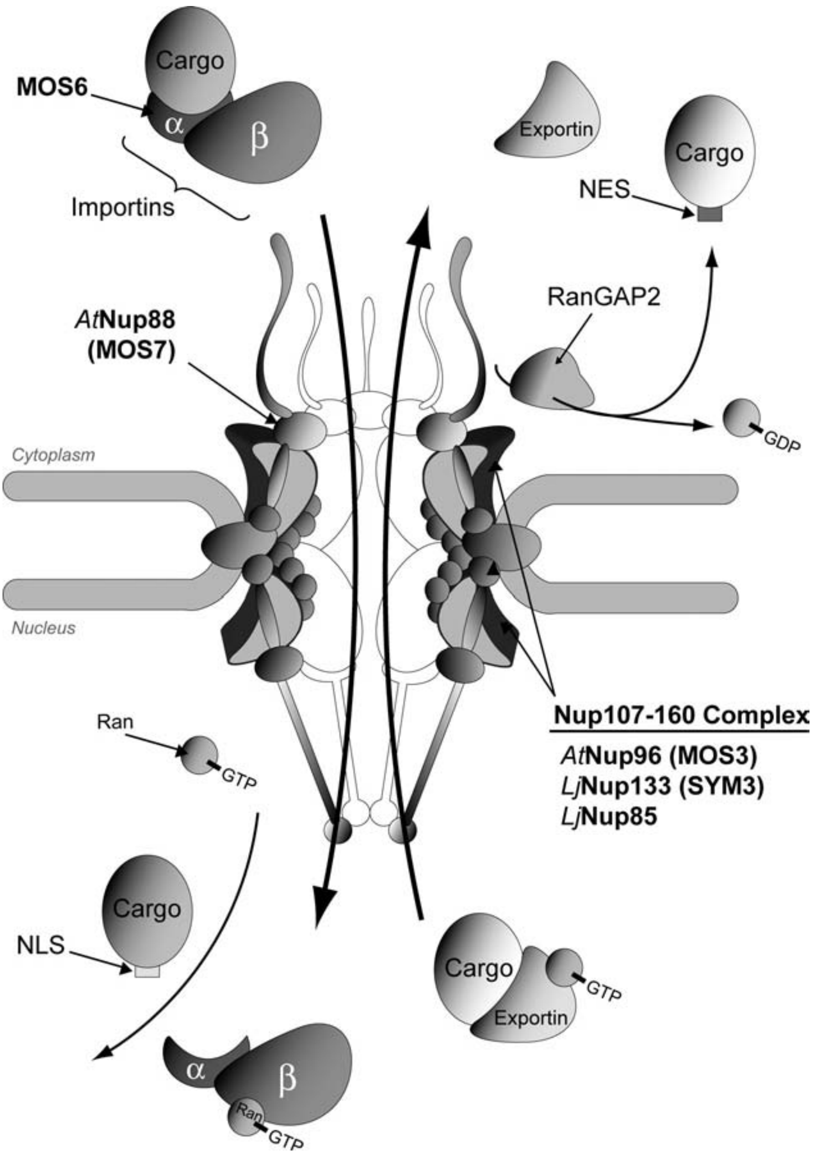

Figure 7.6 Components of the nucleocytoplasmic trafficking machinery implicated in plant–microbe interactions. A schematic cross section of a plant NPC and overview of karyopherin-mediated transport is shown, as described in the text. α, importin-α; β, importin-β; Nup, nucleoporin; RanGAP2, Ran GTPase-activating protein 2. Adapted from Wiermer *et al.* (2007), published by Blackwell Publishing.

EDS1 forms homodimers in the cytoplasm but not the nucleus, where it was shown to interact with SAG101 by FRET (Feys *et al.*, 2005). PAD4 is found in both compartments, where the entire cellular pool likely associates with a small fraction of EDS1, at least in unchallenged cells. The defense regulatory functions of PAD4 and SAG101 are functionally redundant in both basal and TIR-NB-LRR-mediated resistance and HR; indeed, the pathology of *pad4 sag101* double mutants in both types of resistance was more extreme than that of *eds1* mutants (Feys *et al.*, 2005). However, *SAG101* is only partially able to compensate for loss of *PAD4*, implying a unique capability of PAD4, potentially as a consequence of the nuclear restriction of SAG101. In another study discussed earlier, Lipka *et al.* (2005) found that combined *PAD4* and *SAG101* contributions to postinvasion nonhost resistance greatly exceed those of the single components *EDS1*, *PAD4* or *SAG101* alone, supporting the conclusion that PAD4 and SAG101 are partially redundant. Furthermore, genetic additivity was seen between a mutation in the glycosyl hydrolase *PEN2* and a double *pad4 sag101* mutant. While *pen2* mutants exhibited enhanced penetration of nonhost *E. pisi* in *Arabidopsis* with little microcolony formation, penetration rates on *pad4 sag101* double-mutant lines were indistinguishable from those of wild type, yet microcolony formation (resulting from rare successful penetration) was greatly enhanced. In the *pen2 pad4 sag101* triple-mutant lines, the timing and extent of *E. pisi* colonization became indistinguishable from wild-type interactions between *Arabidopsis* and virulent pathogen species, strongly suggesting that PEN2 and the EDS1-PAD4-SAG101 signaling complexes have incremental functions in preinvasion and postinvasion resistance to nonhost powdery mildew fungi (Lipka *et al.*, 2005).

While the molecular role of EDS1 and its partners remains elusive, a study by Wirthmueller *et al.* (2007) found that the strict requirement of TIR-type NB-LRR receptors for EDS1 is not at the level of receptor assembly or distribution in the cell, since RPS4 accumulation and nucleocytoplasmic partitioning were not altered in an *eds1* mutant. To examine the significance of the nucleocytoplasmic distribution of EDS1 complexes, it will be important to localize these complexes in pathogen-induced cells or in autoimmune mutants such as *snc1* or *ssi4*, compared to unchallenged cells. As important defense regulatory proteins that presumably traffic through the NPC, the subcellular distribution of EDS1 and PAD4 in mutants, with defective nuclear import/export machinery (e.g. *mos3*) should also be examined. Real-time imaging technologies can be employed to elucidate stimulus-dependent subcellular localization and interactions of defense regulators in living cells (Koh and Somerville, 2006).

The zinc-finger protein LESION SIMULATING DISEASE RESISTANCE1 (LSD1) negatively regulates an EDS1- and PAD4-dependent cell death pathway and protects plant cells from reactive oxygen-induced stress. *Arabidopsis lsd1* mutants exhibit normal HR after infection by various avirulent pathogens, but runaway cell death is initiated at the margins of these sites (Dietrich *et al.*, 1994). In a prelesion state, *lsd1* mutants have enhanced resistance to several virulent pathogens. Evidence suggests that LSD1 responds

to an ROS signal emanating from the infection site and that it negatively regulates cell death by acting as a cellular redox sensor (Jabs *et al.*, 1996; Dietrich *et al.*, 1997). Results from Rustérucci *et al.* (2001) reveal a genetic link between the disease resistance-promoting functions of *EDS1* and *PAD4* and the negative regulation of plant cell death exerted by *LSD1*, possibly due to EDS1/PAD4-dependent processing of ROS-derived signals. The molecular function of LSD1 was further characterized in a study by Kaminaka and colleagues (2006). In yeast and plant protoplasts, LSD1 antagonizes the nuclear activity of the *Arabidopsis* basic region leucine zipper 10 (bZIP10) transcription factor by sequestering bZIP10 in the cytoplasm. This was not seen when *AtbZIP10* is overexpressed, because LSD1 retention activity is likely titrated out. *At*bZIP10 is a positive regulator of ROS-induced cell death and basal defense, and probably shuttles between the cytoplasm and the nucleus via NLS-dependent nuclear import and NES-dependent nuclear export. Antagonistic functions of LSD1 and *At*bZIP10 modulate disease-resistance responses to an avirulent oomycete (*H. parasitica*) isolate recognized by the R-protein RPP2 presumably via their antagonistic control of host cell death (Kaminaka *et al.*, 2006). A simplified model states that the cytoplasmic *At*bZIP10–LSD1 complex dissociates upon perception of an ROS-derived signal perception and subsequently unmasks the NLS, permitting the nuclear shuttling of *At*bZIP10 and bZIP10-related target gene expression (Kaminaka *et al.*, 2006). So far, the HR- and basal defense-related genes targeted by *At*bZIP10 remain unknown, but the quantitative output in gene expression depends on the relative intracellular amounts of LSD1 and *At*bZIP10, the retention activity of LSD1 and the *At*bZIP10 export rate. It is likely that LSD1 regulates other proteins in addition to *At*bZIP10, as the *lsd1-2 atbzip10* double mutant exhibits the *lsd1* phenotype (Kaminaka *et al.*, 2006).

Redox-regulated translocation from the cytoplasm to the nucleus is an essential feature of another key regulator of the plant pathogen defense, NPR1. The *NPR1* gene encodes a protein containing a bipartite NLS and two protein–protein interaction domains (Cao *et al.*, 1997). Defects in *NPR1* compromise immune responses downstream of SA, including SAR and basal resistance (Cao *et al.*, 1997). In the absence of pathogen infection, NPR1 forms an oligomeric complex in the cytoplasm through intermolecular disulfide bonds (Mou *et al.*, 2003). In response to pathogen attack, an oxidative burst and subsequent biphasic change in the cellular redox environment leads to the reduction of disulfide bonds. NPR1 monomers translocate to the nucleus, possibly due to exposure of normally obscured NLSs, where they regulate downstream *PR* gene expression through interaction with the TGA subfamily of bZIP transcription factors. Mou *et al.* (2003) showed that two critical cysteine residues were essential for NPR1 monomerization. Indeed, a GFP-tagged constitutive monomeric version of the protein with either critical cysteine changed to alanine exhibited an enhanced nuclear accumulation as assayed by fluorescence microscopy and nuclear fractionation experiments. Additional evidence for redox modulation of the interaction between NPR1

and certain TGA TFs inside the nucleus to activate *PR-1* expression was recently reported (Rochon *et al.*, 2006). As discussed at the beginning of this chapter, NPR1 also directly controls the expression of the protein secretory pathway genes through interaction with an unknown TF that binds to the *TL1* element. This element was shown to have a specific protein-binding activity in an electrophoretic mobility shift assay. Intriguingly, when whole-cell extracts were used for this assay, the *TL1*-binding activity was not affected by SA treatment, whereas in nuclear extracts, specific binding was enhanced in SA-treated samples, suggesting that the *TL1*-binding protein also translocates to the nucleus (Wang *et al.*, 2005).

Recent advances in understanding the bridge between cytoplasmic signal transduction and transcription in the nucleus highlight another possible control point based on nucleocytoplasmic partitioning. Pathogen-stimulated MAPK cascades end in transcription of defense-related genes. A MAPK in *Arabidopsis*, MAP KINASE 4 (*At*MPK4), was identified as a negative regulator of SAR (Petersen *et al.*, 2000). *mpk4* mutants exhibit a constitutive SAR phenotype that includes elevated levels of SA, increased resistance to virulent pathogens, and constitutive expression of *PR* genes; the de-regulation of the SA pathway is dependent on EDS1 and PAD4 (Brodersen *et al.*, 2006). Andreasson and colleagues found that GFP-tagged MPK4 and its substrate MAP KINASE 4 SUBSTRATE 1 (MKS1) both localize to the nucleus *in planta* and that MPK4 interacts with WRKY transcription factors implicated in defense gene expression (Andreasson *et al.*, 2005).

Accumulation in the cytoplasm ensures that transcriptional regulators have rapid access to cytoplasmic and PM-resident receptors for activation. The examples of bZIP10-LSD1 and NPR1 demonstrate that spatial restriction of transcription factors or their coactivators by the nuclear envelope is a potent regulatory mechanism utilized by plants to link cytoplasmic signal transduction to rapid defense gene activation in a stimulus-dependent manner. The subcellular localization of MAPK substrates, whether they are TFs or cofactors such as MKS1, might be regulated in a similar manner, although this requires further study. It remains to be determined how nuclear translocation of signal transducers or changes in their nucleocytoplasmic dynamics are mediated upon pathogen perception, and which transport receptors and components of the NPC are involved. Nucleocytoplasmic distribution of some known defense regulatory proteins in the *Arabidopsis* cell is outlined in Fig. 7.7.

7.4 Conclusions

Intracellular trafficking of vesicles to and from the PM and macromolecules across the nuclear pore have emerged as key dynamic processes in all layers of pathogen defense. Mobilization of antimicrobial proteins and compounds to the site of attempted pathogen attack involves concerted cytoskeletal

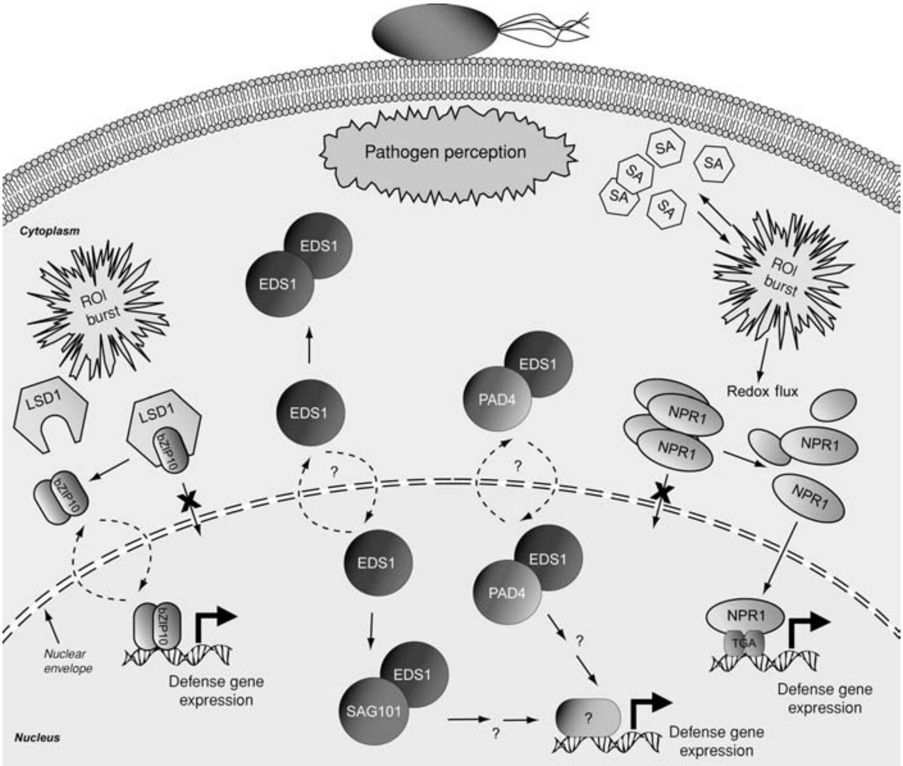

Figure 7.7 Nucleocytoplasmic distribution of known defense regulatory proteins in the *Arabidopsis* cell. The sequestration of the HR-inducing bZIP10 transcription factor in the cytoplasm by LSD1 is released upon pathogen-induced ROS signal perception. Basal defense responses and signaling mediated by TIR type NB-LRR R proteins are dependent on EDS1, PAD4 and SAG101. EDS1 interacts with SAG101 only in the nucleus and forms homodimers exclusively in the cytoplasm of uninduced cells; EDS1 is possibly continuously cycling between the two compartments. EDS1 also interacts with PAD4, and this complex is found in both the nucleus and the cytoplasm. It is unclear whether the nucleocytoplasmic distribution of EDS1 or EDS1–PAD4 changes as a result of pathogen recognition. The defense regulator NPR1 is sequestered as an oligomer in the cytoplasm of un-induced cells. Following pathogen perception, an ROS burst and subsequent shift in redox balance results in an SA-dependent NPR1 monomerization and exposure of its NLS, as described in the text. The NPR1 monomer is translocated to the nucleus where it regulates defense gene expression through its interaction with TGA bZIP transcription factors. ROS, reactive oxygen species; SA, salicylic acid. Reproduced from Wiermer *et al.* (2007), published by Blackwell Publishing.

rearrangements, vesicle transport and fusion, and PM microdomain formation. While some genes involved in these events have been isolated, much work remains to elucidate the molecular mechanisms of regulation.

Molecular genetic studies in *Arabidopsis*, barley and tobacco show that proteins involved in vesicle fusion are integral to penetration resistance to

nonhost fungal pathogens, and that several of these are phosphorylated in response to PAMPs and may control other defense-signaling pathways. In addition, the seven-transmembrane MLO family of proteins is likely negative regulators of exocytotic processes. Other *PEN* genes encode a putative ABC transporter and peroxisomal glycosyl hydrolase. All of these proteins localize to infection sites either in lipid rafts on the PM or in accumulated vesicles and organelles. The actin cytoskeleton likely plays a pivotal role in this trafficking. Furthermore, endocytosis of the FLS2 receptor in response to flg22 suggests that internalization of surface receptors is a probable mechanism in other PAMP- and R-protein-mediated responses. Both exocytosis/secretion and endocytosis require dynamic movement of regulators to and from infection sites. Several key questions remain. What are the antimicrobial compounds and peptides secreted into the apoplast and what is their specificity to certain pathogens? PEN2 and PEN3 might be involved in the secretion of antimicrobial toxins – metabolic profiling of secondary metabolites of different compounds missing in *pen2* and *pen3* mutants compared to wild type may yield informative data on the roles of these proteins. Similarly, an expression proteomics approach can be utilized to examine differentially secreted peptides in *pen1* versus wild type in response to pathogen attack. How does MLO regulate vesicle fusion events? Preliminary data suggest that AtMLO2 interacts with PEN1. Since syntaxins exhibit N-terminal auto-inhibitory domains, it will be important to determine whether N-truncated PEN1 phenocopies the *Atmlo2* mutant phenotype. What is the role of syntaxins in the negative regulation of SA-dependent effector-mediated resistance and HR, processes that do not appear to rely on secretion? Suppressor screens in the *syp121/syp122* background should identify downstream targets of negative regulation. Alternatively, intracellular build up of antimicrobial compounds could trigger inappropriate HR. Besides HopM1, do other pathogen effectors specifically interfere with components of vesicular transport or fusion? Great efforts are being made to clone and characterize additional pathogen effectors. Protein interaction data may yield further insights into this open question. Since these processes are critical for multiple layers of defense, we anticipate that regulators of focal secretion/exocytosis are candidates for manipulation by pathogen virulence effector function. Is endocytosis of surface pathogen receptors a general phenomenon in plant defense signaling? While endocytosis of FLS2 occurs during an immune response, is this also a feature of other plasma membrane receptors, such as the R proteins Cf4 and Cf9 in tomato, that possess a conserved endocytosis signature at their C-terminus? Recent advances in live-cell fluorescence imaging could help answer this question. What is the contribution of the rest of the secretory pathway in defense? Further genetic, cellular and biochemical analyses should reveal more players in upstream or early events of vesicle trafficking.

Genetic analysis in *Arabidopsis* has shown that defects in the nuclear trafficking machinery suppress multiple layers of defense. Recent work in barley, tobacco and *Arabidopsis* has identified intracellular NB-LRR R-protein

receptors in the nucleus and that their nuclear pools are essential for defense signaling. Other defense regulators, such as NPR1, LSD1, MKS1, EDS1 and SAG101, exhibit nuclear trafficking and, as with NPR1 and LSD1, may be regulated by nucleocytoplasmic partitioning. What are the roles of nuclear and cytoplasmic R-protein pools and how are the composition and distribution of R-protein complexes regulated? Moreover, do autoimmune mutants, such as *snc1*, exhibit R-protein mis-localization? Is the localization of R proteins or other known defense regulators (e.g. EDS1) affected by defects in the nuclear trafficking machinery? Again, detailed biochemical analysis coupled with live-cell fluorescence imaging after pathogen elicitation is probably key to determining changes in nuclear versus cytoplasmic concentrations of R proteins and other defense regulators. Novel regulators of R-protein function, discovered in suppressor and interaction screens, may play a role in nucleocytoplasmic localization of R-protein-containing complexes. Proteomics of defense regulator complexes isolated from nuclei of infected versus noninfected cells might illuminate this phenomenon. Do pathogen effectors specifically interfere with nuclear transport to promote virulence? Previously, several pathogen effector proteins were implicated in direct or indirect interaction with components of the nuclear transport machinery, for example, AvrBS3 with α-importin. This has been interpreted as evidence for hijacking of the host nuclear transport machinery for nuclear import. It is possible that manipulation of the nuclear import machinery promotes pathogen virulence. New data showing that the Rx interaction with RanGAP2 is required for recognition of PVX CP suggest that RanGAP2 may be a target of the effector. In light of the importance of dynamic nucleocytoplasmic regulation in pathogen defense, we expect some newly characterized pathogen effectors may target this process.

In conclusion, exocytosis, endocytosis and nucleocytoplasmic trafficking in pathogen defense are emerging research fields in plant science – future studies that address these and other fundamental questions will provide important insights into eukaryotic innate immunity.

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Chapter 8

ROLE OF PLANT SECONDARY METABOLITES AT THE HOST–PATHOGEN INTERFACE

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Abstract: Plants synthesise and accumulate a large number of structurally diversified small molecules, usually derived from amino acids or some other end products or intermediates of plant primary metabolism. Some groups of these 'secondary metabolites' possess in vitro antimicrobial activities, typically through inhibition of essential microbial enzyme activities or disintegration of cellular compartments. This suggests a potential functional significance of plant secondary products as executors of plant immune responses. Consistent with this, impairment of the capacity of pathogens to metabolise/detoxify such compounds often increases virulence. Defence-related phytochemicals are often classified on the basis of their in planta mode of biosynthesis and accumulation as phytoalexins or phytoanticipins. Their synthesis and/or activation are typically coordinated in time and space with other defence responses upon microbial colonisation attempts. Single plant cells are capable to target resistance responses with subcellular precision to incipient microbial entry sites. One function of this process appears to enable high local concentrations of toxic metabolites. Traditional plant breeding revealed that disease resistance correlates in some cases positively with the ability of plants to synthesise particular secondary products. This is corroborated by transgenic plants that fail to either accumulate phytoalexins or synthesise novel pathogen-inducible toxic metabolites.

Keywords: antimicrobial activity; detoxification; metabolic engineering; phytoalexins; phytoantycipins; secondary metabolites

8.1 Introduction

Plants have evolved the capability to synthesise and accumulate an unusually large number of structurally diversified small molecules known as secondary

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metabolites. Different compound classes are often derived from different precursor molecules. In most cases, these precursors are amino acids or some other end products or intermediates of plant primary metabolism (Fig. 8.1). The occurrence of particular plant natural products is usually restricted to narrow phylogenetic lineages such as a family or genus and is in some cases even limited to a single species. This suggests that natural chemical diversity in plants is probably the result of genetic adaptation mechanisms to environmental cues, including responses to biotic and abiotic stresses.

A subset of plant secondary products was known for many years to have antimicrobial properties. Traditionally used for alleviating illness in folk medicine, plant-derived compounds are nowadays considered as a useful source of templates for novel antibiotics (Lewis and Ausubel, 2006). The potential involvement of antimicrobial plant metabolites in plant defence against microbial invaders is one of the longest studied plant responses to pathogens (Link *et al.*, 1929; Müller and Börger, 1940). Evidence gathered over the last decades strongly supports the idea that biosynthesis and accumulation of antimicrobial molecules is one of the evolutionary conserved responses of flowering plants to pathogenic microorganisms (Hammerschmidt, 1999; Mansfield, 2000; Dixon, 2001). Thus, the regulatory principles appear to be conserved whilst the biosynthetic products are structurally diverse.

Because of the evolutionary conserved role of secondary metabolites in plant defence, it is plausible that plants integrated control of the corresponding biosynthetic machinery as well as cellular mechanisms for compound storage and delivery into the plant immune system. Thus, the engagement of secondary metabolites in microbial defence must be coordinated with a multitude of other cellular reprogramming events initiated upon perception of non-self-structures by plant immune receptors (see Chapters 2 and 10). The structural diversity of secondary products in different plant species in encounters with microbial parasites is a feature that distinguishes the plant from the animal immune system and suggests that the capacity for chemical diversification is evolutionarily driven by parasites. Considering the enormous structural complexity of plant secondary metabolites, we discuss here mainly selected compound classes for which combined biochemical and genetic evidence points to infection-limiting functions. With this in focus, we refer to other reviews covering the enzymatic machinery of secondary metabolites as well as the coordination of secondary metabolism with plant developmental programmes (Dixon and Paiva, 1995; Sicker *et al.*, 2000; Halkier and Gershenzon, 2006; Glawischnig, 2007).

8.2 Classification of defence-related plant secondary metabolites

Antimicrobial plant metabolites can be classified according to different criteria, for example the biosynthetic origin of a compound of interest. Since

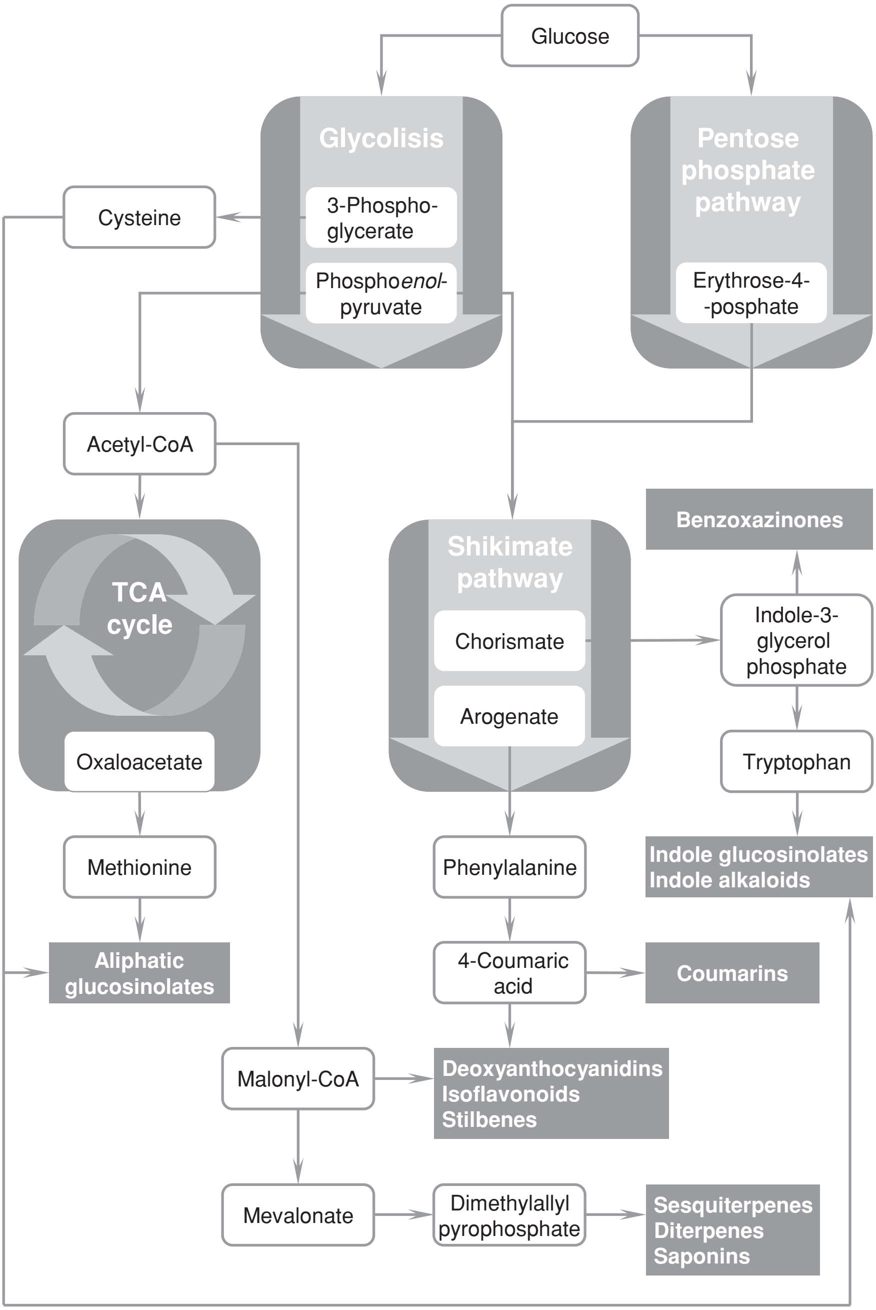


Figure 8.1 Biosynthetic connections between primary metabolism and selected classes of plant secondary products (listed in shaded rectangle boxes).

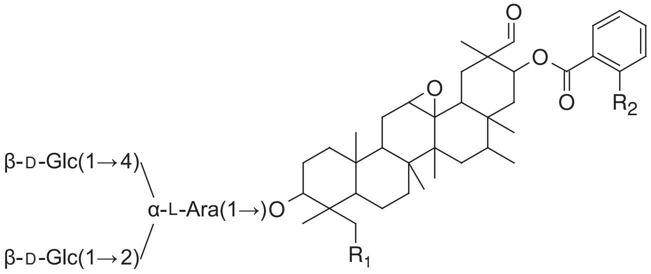
a purely chemical classification can obscure potential in planta regulatory commonalities, a classification based on the mode of biosynthesis and accumulation of defence-related phytochemicals was introduced. Accordingly, defence-related compounds constitutively stored or released from constitutive precursors are called phytoanticipins (VanEtten *et al.*, 1994) while those synthesised de novo in response to microbial infection are designated phytoalexins (Müller and Börger, 1940; Paxton, 1981).

8.2.1 Phytoanticipins

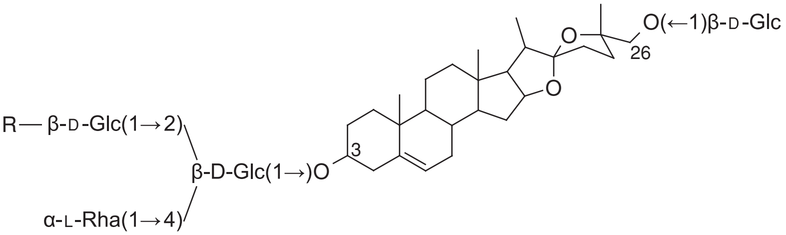
Phytoanticipins are defined as antimicrobial compounds that are either preformed or become released from constitutively generated precursors following microbial invasion (VanEtten *et al.*, 1994). A large number of different secondary plant products accumulate constitutively in plant cells and many were reported to have in vitro antimicrobial activity. However, only for a small subset of these compounds are there in vivo data to support a role in growth restriction of microbial pathogens. Here, we concentrate on three classes of constitutive secondary products: saponins, benzoxazinone glucosides and glucosinolates.

Saponins are glycosides of triterpenes, steroids or steroidal alkaloids, widely occurring in both mono- and dicotyledonous plant species (Osbourn, 1996). The best-studied examples of compounds belonging to this metabolic group are steroidal and triterpenoid saponins from oats (*Avena* spp.), which became a model system for investigating their biosynthesis as well as functions in plant–microbe interactions (Osbourn *et al.*, 1994; Papadopoulou *et al.*, 1999). Most tested oat species accumulate, in a mutually exclusive manner, two groups of saponins: triterpenoid avenacins A-1, A-2, B-1 and B-2 in roots (Fig. 8.2; Maizel *et al.*, 1964; Crombie *et al.*, 1986a) and steroidal avenacosides A and B in leaves (Fig. 8.2; Tschesche *et al.*, 1969; Tschesche and Lauven, 1971). Besides differences in the aglycone backbone, the two groups also differ in the sugar substitution pattern. Avenacins belong to monodesmosidic saponins; they have an oligosaccharide chain only at the C-3 position, while avenacosides are bisdesmosidic, i.e. have an additional glucose moiety attached at the C-26 position (Fig. 8.2). Apart from oat saponins, the functions of steroidal glycoalkaloids were also examined in solanaceous plant species during plant–microbe interactions. A well-studied example of a Solanaceae secondary metabolite is α-tomatine (Fig. 8.2), a major saponin of tomato (*Lycopersicon esculentum*; Arneson and Durbin, 1968). Already 50 years ago, avenacins and α-tomatine were reported to inhibit the growth in vitro of a wide range of pathogenic as well as non-pathogenic fungi and oomycetes (Maizel *et al.*, 1964; Arneson and Durbin, 1968), prompting experiments to test the physiological significance of their antimicrobial activity (see below).

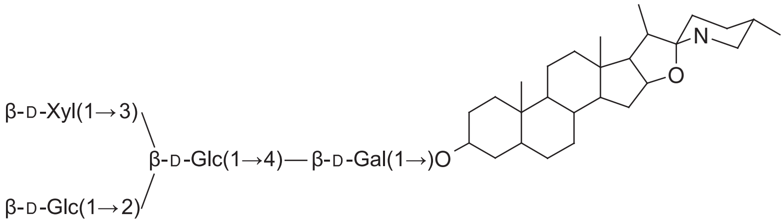
A second group of constitutive secondary metabolites that are postulated to exert rate-limiting functions in plant defence against parasites are benzoxazinone glucosides. These compounds are found in some members of the grass



Avenacin A-1: R_1 = OH; R_2 = $NHCH_3$ Avenacin A-2: R_1 = OH; R_2 = H
Avenacin B-1: R_1 = H; R_2 = $NHCH_3$ Avenacin B-2: R_1 = H; R_2 = H



Avenacoside A: R = H Avenacoside B: R = β-D-Glc(1→3)



α-Tomatine

Figure 8.2 Structures of selected saponins. C-3 and C-26 carbons are labelled for avenacosides.

family (Gramineae), including maize (*Zea mays*), wheat (*Triticum* spp.), rye (*Secale cereale*) and certain wild, but not cultivated, barley (*Hordeum*) species (Sicker *et al.*, 2000). Rye and wild barley mainly accumulate the glucoside of 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA; Fig. 8.3), while in maize and wheat the major compound from this class is the glucoside of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA; Fig. 8.3). Accumulation of

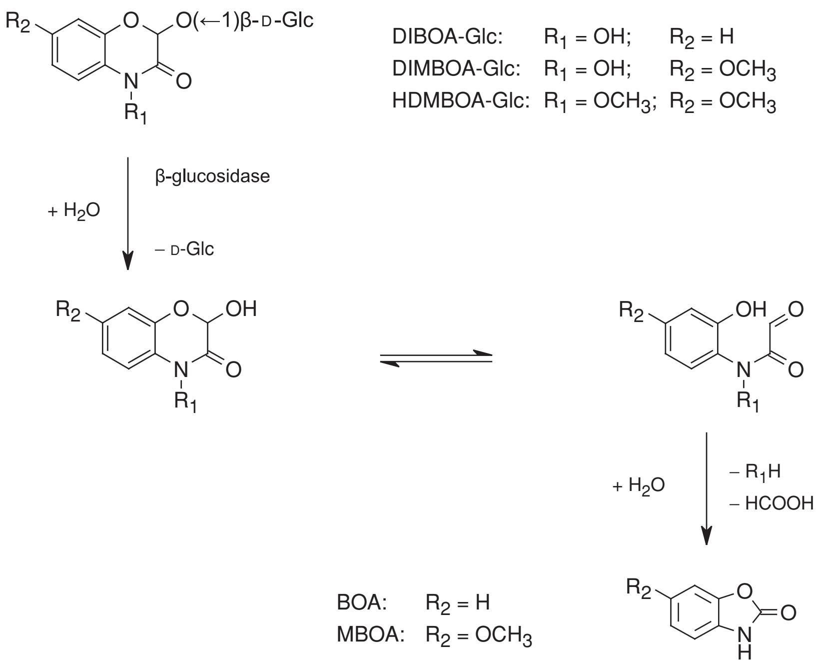


Figure 8.3 Examples of benzoxazinone glucoside structures and their β-glucosidase triggered degradation. DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; HDMBOA, 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one; BOA, 2-benzoxazolinone; MBOA, 6-methoxy-2-benzoxazolinone.

benzoxazinones in plant tissue was shown to be under developmental control: it is usually highest in young seedlings and decreases with increasing plant age (Argandona and Corcuera, 1985). Benzoxazinone glucosides were proposed to contribute to disease resistance responses based on the positive correlation between their accumulation in plant tissue and resistance against various pathogens (Niemeyer, 1988). Benzoxazinone aglycones confer antifungal activity in vitro against a range of phytopathogenic fungi including *Gaeumannomyces graminis*, *Fusarium moniliforme*, *Fusarium culmorum* and *Helminthosporium turcicum* (Couture *et al.*, 1971; Friebe *et al.*, 1998), and were also shown to affect the virulence of *Agrobacterium tumefaciens* (Zhang *et al.*, 2000).

The third group of compounds are glucosinolates (Fig. 8.4) – unique nitrogen- and sulphur-containing plant secondary metabolites reported almost exclusively in the order Capparales, which includes agriculturally important crop plants of the Brassicaceae family (Grubb and Abel, 2006; Halkier and Gershenzon, 2006). The glucosinolate core structure is derived from amino acids, including methionine, tryptophan and phenylalanine, and

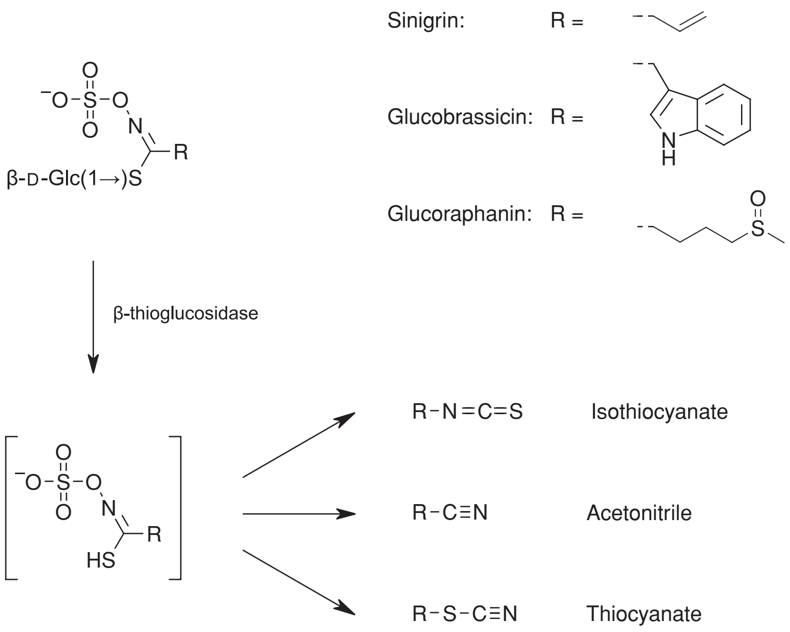


Figure 8.4 Representative structures of glucosinolates and their myrosinase (β-thioglucosidase) triggered degradation. Only some of the possible end products are shown as examples. Formation of respective degradation products is dependent on the R group, pH and the presence of additional proteins.

comprises a β-thioglucosyl residue. Compounds belonging to this metabolite group are usually stable in vivo. However, loss of cellular integrity initiates rapid glucosinolate breakdown by myrosinase (β-thioglucosidase)-catalysed hydrolysis of the glucosidic bond. Subsequent to the enzymatic hydrolysis, non-enzymatic reactions lead to the formation of a variety of bioactive products (Fig. 8.4), of which at least some are likely to contribute to plant defence against generalist herbivores (Halkier and Gershenzon, 2006). The same class of degradation products were proposed to act in plant interactions with microbial organisms, but this is based primarily on in vitro antimicrobial properties (Mithen *et al.*, 1986; Brader *et al.*, 2001).

8.2.2 Phytoalexins

The term phytoalexin was proposed by Müller and Börger (1940) for a hypothetical defensive substance induced in potato tuber tissue by an avirulent strain of the late blight oomycete parasite *Phytophthora infestans*. The presence of this factor was inferred from the failure of a virulent *P. infestans* isolate to colonise tissue pretreated with the avirulent strain. The initial definition of phytoalexins underwent some revisions and was finally used to describe

low-weight antimicrobial metabolites that are synthesised and accumulated in plants after pathogen challenge (Paxton, 1981). To date, many phytoalexins have been isolated from different species (Grayer and Harborne, 1994; Hammerschmidt, 1999; Harborne, 1999). Apparently, each examined plant species is capable of synthesising de novo antimicrobial compounds upon attempted microbial invasion, suggesting that this capacity is evolutionary ancient and indispensable.

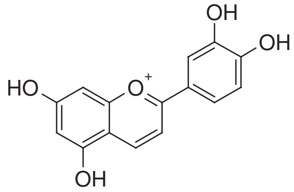
Many phytoalexins are derived from phenylalanine (Fig. 8.5a; Dixon and Paiva, 1995). These include 3-deoxyanthocyanidins that were reported to accumulate after pathogen challenge in sorghum (*Sorghum bicolor*; Snyder and Nicholson, 1990) as well as pterocarpan-type isoflavonoid phytoalexins that are characteristics for some Leguminosae species. Examples of the latter class of metabolites are phaseollin synthesised by bean (Perrin, 1964), pisatin produced by pea (*Pisum sativum*; Perrin and Bottomley, 1961), glycinol reported as a phytoalexin in soybean (*Glycine max*; Weinstein and Albersheim, 1983) and medicarpin from alfalfa (*Medicago sativa*; He and Dixon, 2000). Coumarins constitute another class of phenylalanine-derived phytoalexins including scopoletin, a pathogen-inducible metabolite of rubber tree (*Hevea brasiliensis*; Churngchow and Rattarasarn, 2001), tobacco (*Nicotiana tabacum*; Costet *et al.*, 2002) and some of the *Heliantus* spp. (GutierrezMellado *et al.*, 1996; Cabello-Hurtado *et al.*, 1998), as well as furanocoumarins (e.g. bergapten) in parsley (*Petroselinum crispum*; Parker *et al.*, 1991).

Of particular interest are phenylalanine-derived stilbenes, with resveratrol as the major representative. Resveratrol is pathogen-inducible in several phylogenetically unrelated species like grapevine (*Vitis vinifera*; Langcake and Pryce, 1976) or peanut (*Arachis hypogaea*; Sobolev *et al.*, 1995). This compound is synthesised by stilbene synthase (STS), which is closely related to chalcone synthase (CHS) and belongs, like the former enzyme, to the family of polyketide synthases. Phylogenetic analysis of plant-specific CHSs and STSs as well as structure–activity relationships of variants of a synthetic inter-family hybrid protein suggest that STSs evolved several times independently from CHSs (Tropf *et al.*, 1994). One possibility is that lineage-specific selection pressure by parasites has driven this evolutionary trajectory (see below).

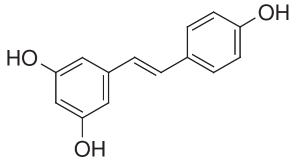
Terpene-type phytoalexins (Fig. 8.5b) have been described in cotton (*Gossypium* spp.). In interactions with *Xantomonas campestris*, a series of sesquiterpenoid compounds accumulate of which 2,7-dihydroxycadalane was found to be the most toxic (Essenberg *et al.*, 1992). Terpene-type antimicrobial metabolites are also pathogen-inducible in rice (*Oryza sativa*). This species produces a family of labdane-related diterpenoid phytoalexins including momilactones, oryzalexins and phytocassanes (Peters, 2006).

Brassicaceae plant species accumulate sulphur-containing tryptophan-derived alkaloid phytoalexins (Fig. 8.5c; Pedras *et al.*, 2007). One of the best studied of these is camalexin (Glawischnig, 2007), which is produced in response to microbial infection by the reference plant *Arabidopsis* (Tsuji *et al.*, 1992) and some other closely related species (Browne *et al.*, 1991; Jimenez *et al.*,

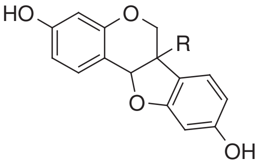
(a)



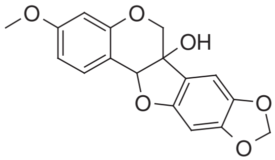
3-Deoxyanthocyanidin



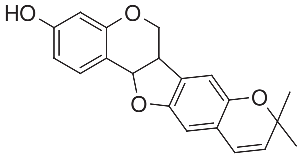
Resveratrol



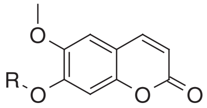
Glycinol: R = OH
Medicarpin: R = H



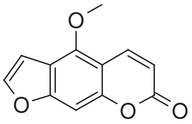
Pisatin



Phaseollin

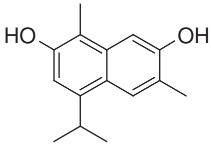


Scopoletin: R = H
Scopolin: R = β-D-Glc(1→)

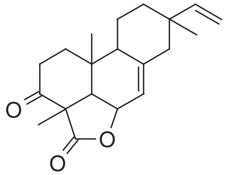


Bergapten

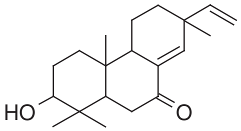
(b)



2,7-Dihydroxycadalene

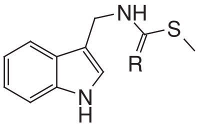


Momilactone A

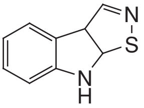


Oryzalexin A

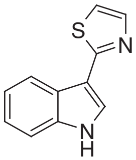
(c)



Brassinin: R = S
Brassitin: R = O



Brassilexin



Camalexin

Figure 8.5 Structures of selected phytoalexins representing different classes of plant secondary products: (a) phenylpropanoids, (b) terpenes and (c) sulphur-containing indole alkaloids.

1997; Zook *et al.*, 1998). The biosynthesis and in vivo function of camalexin in *Arabidopsis* defence reactions were studied in greater detail (Glazebrook and Ausubel, 1994; Thomma *et al.*, 1999; Glawischnig *et al.*, 2004; Nafisi *et al.*, 2007). One long-term objective of these studies is to generate tools for rational modification of secondary metabolite pathways in economically relevant Brassicaceae species with improved disease resistance. Besides camalexin, several other tryptophan-derived metabolites, mainly derivatives of indole-3-carboxylic acid, are induced in *Arabidopsis* after pathogen attack (Hagemeier *et al.*, 2001; Tan *et al.*, 2004; Bednarek *et al.*, 2005). Evidence for their contribution to plant defence responses is still lacking.

8.2.3 Phytoanticipin or phytoalexin – a flexible borderline

Since the difference between phytoanticipins and phytoalexins is based on the mode of biosynthesis and accumulation of a particular compound, the same compound type can be assigned to both classes if regulatory innovations contributed to the diversification of a metabolite pathway (VanEtten *et al.*, 1994). Scopolin, a glucose conjugate of scopoletin, constitutively accumulates to high levels in *Arabidopsis* roots (Bednarek *et al.*, 2005; Kai *et al.*, 2006a), but is inducible in leaves upon infiltration with the bacterial pathogen *Pseudomonas syringae* (P. Bednarek and M. Bartsch, unpublished data).

A strict division between constitutive and inducible plant secondary products is also sometimes difficult since some constitutive metabolites undergo modifications that are induced by pathogen challenge. For example, DIMBOA glucoside is converted into a glucoside of 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA; Fig. 8.3) in maize following challenge with adapted parasitic fungi including *Bipolaris maydis* or *Curvularia lunata* or non-adapted *Alternaria alternata* (Oikawa *et al.*, 2004). Likewise, some tryptophan-derived glucosinolates that are likely produced by inducible biosynthesis were identified in *Arabidopsis* leaves and roots upon treatment with culture filtrates of *Erwinia carotovora* or challenge with *Pythium sylvaticum*, respectively (Brader *et al.*, 2001; Bednarek *et al.*, 2005).

8.3 Biological activity of plant secondary metabolites

Many plant-derived small molecules show in vitro antimicrobial activity against a broad range of potential plant pathogens, prompting questions on the underlying biochemical mechanism(s). Clearly, understanding the in vivo activity of particular metabolites requires knowledge of their direct target(s).

8.3.1 Mode of action

Although structural information on phytoanticipins and phytoalexins has been available for many years, surprisingly little is known about the mode

of action of these compounds. Frequent targets appear to be biomembranes, crucial cell components that insulate reactive components from each other in different cellular compartments. Membranolytic activity has been assigned to saponins and was studied in vitro in greater detail. Model saponins, including α-tomatine and avenacine A-1, can disrupt both artificial as well as natural lipid bilayers (Steel and Drysdale, 1988; Keukens *et al.*, 1995; Armah *et al.*, 1999). The aglycone part of saponins is thought to insert into lipid bilayers and subsequently preferentially associate with membrane-resident sterol residues. In a second step, the glycone part of saponins can lead to reorganisation of sterols in the target membrane, finally leading to the formation of pores (Fig. 8.6; Keukens *et al.*, 1995; Armah *et al.*, 1999). The membranolytic activity of saponins is dependent on the presence of sterols with a free 3β-hydroxy group in the target membrane (Keukens *et al.*, 1995; Steel and Drysdale, 1988; Armah *et al.*, 1999) as well as on an intact sugar moiety at the C-3 position of the saponin aglycone (Keukens *et al.*, 1995; Armah *et al.*, 1999). The complexity of saponin activity was revealed when budding yeast (*Saccharomyces cerevisiae*) was employed as model eukaryotic target cell (Simons *et al.*, 2006). The glycoalkaloid α-tomatine has antifungal activity towards yeast. This activity is associated with membrane permeabilisation and requires the presence of the tetrasaccharide chain. However, complete removal of the sugar side chain enhanced antifungal activity. It is possible that the activity of the aglycone

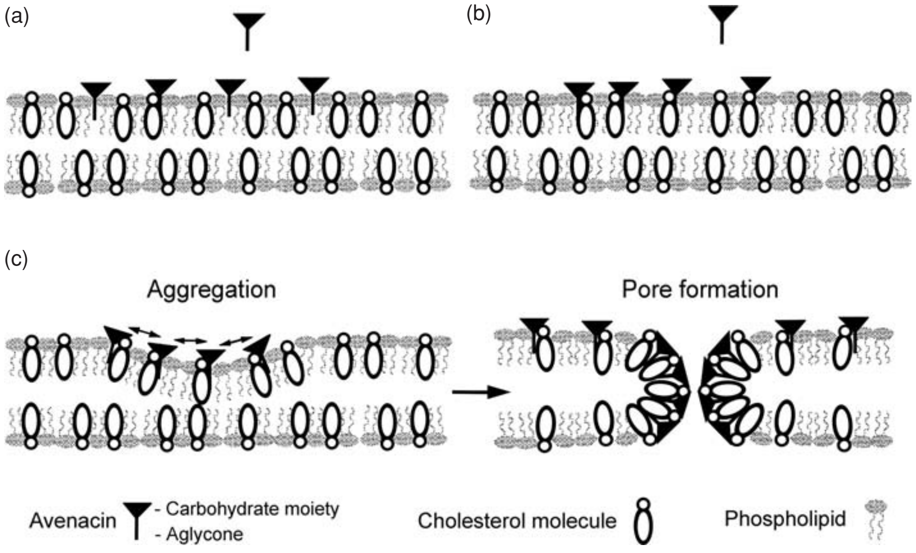


Figure 8.6 The proposed model for pore formation by avenacin A-1: (a) cholesterol-independent insertion of the aglycone portion of avenacin A-1 into the cis leaflet of the membrane, (b) binding of cholesterol to avenacin A-1 in the membrane (c) and formation of the transmembrane pore. (Reproduced from Armah *et al.* (1999), by permission of The Biophysical Society.)

tomatidine does not involve membrane permeabilisation, but inhibition of ergosterol biosynthesis since there is accumulation of zymosterol rather than ergosterol. However, *erg6* and *erg3* yeast mutants exhibit enhanced resistance to tomatidine, indicating a complex interplay between *erg* mutations, sterol content and tomatidine resistance.

The plasma membrane and/or endomembranes are most likely also a target for some phytoalexins. The mode of action of some of the pterocarpan-type isoflavonoids, including phaseollin, glycinol and pisatin, has been linked with direct membrane disruption or indirect effects on processes that are important for membrane function (VanEtten and Bateman, 1971; Shiraishi *et al.*, 1975; Weinstein and Albersheim, 1983). For example, sorghum-derived 3-deoxyanthocyanidins showed the ability to disintegrate lipid bilayers in vitro (Nielsen *et al.*, 2004), whilst *Arabidopsis* camalexin was capable of disrupting bacterial membranes when the compound was tested against the phytobacterium *P. syringae* pv. *maculicola* (Rogers *et al.*, 1996). A screen for camalexin-resistant mutants of *P. syringae* pv. *maculicula* yielded exclusively partially resistant mutants that were also less sensitive to tetracycline and nalidixic acid. This hints at components of the outer bacterial membrane contributing to toxin sensitivity.

Other phytochemicals apparently do not target lipid membranes. For example, benzoxazinone aglycones were shown to directly affect the activity of a wide range of enzymes including papain (Perez and Niemeyer, 1989b), α-chymotrypsin (Cuevas *et al.*, 1990), glutathione S-transferase (Sant'anna *et al.*, 2002) and the plasma membrane-resident H^+-ATPase (Friebe *et al.*, 1997). The biochemical activity of benzoxazinone aglycones is linked to their intrinsic instability. Some of the observed enzyme inhibitory effects are attributed to the ring chain tautomeric aldehyde, which is proposed to act as an electrophile in reactions with protein nucleophiles. For example, DIMBOA forms in vitro imine adducts with the NH_2 group of *N*-acetyl lysine (Perez and Niemeyer, 1989a). The inhibition of α-chymotrypsin results from addition of the aldehyde to the catalytic serine (Cuevas *et al.*, 1990). Likewise, inhibition of glutathione S-transferase activity is thought to involve the reaction of the tautomeric aldehyde with the thiol residue of reduced glutathione (Sant'anna *et al.*, 2002). A further complication is that parts of the activity of benzoxazinones are the result of their metabolic modification: the O-acylated derivatives could act as alkylating agents towards proteins and nucleic acids, which could explain their mutagenic activity (Ishizaki *et al.*, 1982; Hashimoto and Shudo, 1996).

In addition to antimicrobial activities of plant metabolites, a subset may have dual action by modulating the activity of plant-derived defence signalling molecules. Such a role is likely played by scopoletin, which, apart from its direct antimicrobial activity (Churngchow and Rattarasarn, 2001), appears to act as buffer of reactive oxygen species (ROS) that are produced upon pathogen challenge (Chong *et al.*, 1999, 2002). A reduction of scopoletin and scopolin levels was observed in transgenic tobacco plants in which

antisense constructs downregulated the expression of genes encoding two pathogen-inducible UDP-Glc:phenylpropanoid glucosyltransferases (UGTs). These UGTs efficiently glycosylate the hydroxycoumarin scopoletin and hydroxycinnamic acids. The UGT-depleted lines exhibited weakened resistance responses to tobacco mosaic virus but sustained accumulation of ROS at infection foci. The latter phenomenon is thought to reflect decreased ROS-scavenging activity because of reduced scopoletin levels (Chong *et al.*, 2002). Similarly, DIMBOA was shown to enhance the rate of horseradish peroxidase isoenzyme C-catalysed NADH oxidation, leading to the production of hydrogen peroxide (Rojas *et al.*, 1997). The latter ROS is known to act as a signalling molecule during plant defence responses (Laloi *et al.*, 2004). However, it is not clear if this stimulatory effect of enzymatic activity is physiologically relevant.

8.3.2 Plant self-protection mechanisms

How do plants avoid the toxicity of their own biologically active products? Self-protection seems to be important since some plant-derived antimicrobials have phytotoxic properties (Rogers *et al.*, 1996; Steel and Drysdale, 1988; von Rad *et al.*, 2001). One way to prevent self-poisoning is to synthesise bioactive compounds only after pathogen challenge and to restrict the synthesis precisely to sites of attempted infection. Whilst these are typical features of phytoalexins, phytoanticipins or their precursors are synthesised constitutively and accumulate in plant tissue without recognisable adverse effects. One potential self-protection mechanism involves the modification of endogenous biomolecules that are metabolite targets. For example, the membrane-disrupting ability of saponin model compounds is strictly dependent on the presence of sterols with a free 3β-hydroxy group in the target membrane (Keukens *et al.*, 1995; Armah *et al.*, 1999). It is notable here that at least some saponin-producing Solanaceae plants, including tomato, contain exceptional levels of substituted sterols (Duperon *et al.*, 1984). The concomitant reduction in sterols with a free alcohol group correlates with enhanced resistance of tomato and potato tissue against α-tomatine-induced electrolyte leakage, an indicator of plasma membrane integrity (Steel and Drysdale, 1988). This could explain why tomato and potato withstand high concentrations of saponins.

Another means by which plants try to control their own chemical weapons is storage of active molecules in the form of inactive conjugates or precursors and compartmentalisation away from the respective activating enzymes. Many plant secondary metabolites accumulate inside cells as glycosides, frequently as glucose conjugates (glucosides). This is a common feature of most known phytoanticipins (Sicker *et al.*, 2000; Halkier and Gershenzon, 2006), but applies also to some pathogen-inducible compounds, for instance scopoletin (Costet *et al.*, 2002). Attachment of a polar sugar residue to the usually unpolar aglycone is thought to be important for transport and storage. Also, since

glycosylation typically leads to a reduction of biological activity, it is likely a means by which plants protect themselves from their phytotoxic properties. However, this generalisation is not always valid since the sugar chain in the C-3 position of saponin aglycones is an indispensable component of their biological activity (Keukens *et al.*, 1995; Armah *et al.*, 1999). By contrast, the bisdesmosidic saponins like avenacosides are constitutively inactive due to the presence of a glucose moiety at the C-26 position of the aglycone. These compounds can be activated by a specialised β-glucosidase, which releases biologically active 26-desavenacosides (Gus-Mayer *et al.*, 1994). The enzyme accumulates inside plastids (Nisius, 1988), whilst avenacosides localise predominately to vacuoles (Kesselmeier and Urban, 1983), suggesting that the enzyme is spatially separated from its substrate in intact cells. Cleavage of the glucoside leading to the release of an active aglycone moiety is thought to occur upon disruption of cellular integrity by destructive pathogens.

Benzoxazinones accumulate in planta mainly as glucosides (Sicker *et al.*, 2000). Two glucosyltransferases, designated BX8 and BX9, are involved in the synthesis of benzoxazinone glucosides (von Rad *et al.*, 2001). It is notable that expression of both genes in *Arabidopsis* rendered the transgenic lines more resistant to the phytotoxic activity of DIBOA and DIMBOA (von Rad *et al.*, 2001), supporting the idea that their glucosylation can serve as a self-protection mechanism. Benzoxazinone glucosides accumulate in vacuoles (Sicker *et al.*, 2000), whilst the corresponding β-glycosidase localises to plastids (Babcock and Esen, 1994). Thus, the principle of compartmentalising benzoxazinone glucosides away from its activating enzyme appears to be the same as for avenacosides. The β-glycosidase-mediated activation of constitutive benzoxazinone glucosides is postulated to occur following disintegration of organelles of attacked plant cells. Upon cleavage, DIBOA and DIMBOA are unstable in vitro and decompose to benzoxazolinones, 2-benzoxazolinone (BOA) and 6-methoxy-2-benzoxazolinone (MBOA), respectively, with the concomitant release of formic acid (Fig. 8.3; Woodward *et al.*, 1978).

The glycosylation and de-glycosylation steps are also of importance for the biological function(s) of glucosinolates. These sulphur-containing compounds are supposed to localise to vacuoles. Insight into their localisation was obtained by energy dispersive X-ray imaging of *Arabidopsis* flower stalks. This revealed sulphur-rich cells (S cells) located mainly between vascular bundles and the endodermis (Koroleva *et al.*, 2000). Subsequent biochemical analyses of cell sap collected from S cells showed high levels of glucosinolates, confirming that S cells accumulate high levels of glucosinolates. In intact plant organs, such as leaves, glucosinolates are compartmentalised from specific β-thioglucosidases (myrosinases), which in turn accumulate in specialised myrosin cells. In *Arabidopsis*, these cells were found in the phloem parenchyma adjacent to S cells using immunocytochemical analyses, while in *Brassica napus* myrosin cells were also present in ground tissue (Andreasson *et al.*, 2001). This might serve as an example of how cell-type-specific

compartmentalisation of a 'pro-toxin' and its activating enzyme prevent detrimental effects in healthy plants. However, subcellular immunolocalisation of both glucosinolate (sinigrin) and myrosinase in cotyledons of *Brassica juncea* seedlings showed that these two components also co-localise in some cells, suggesting the existence of an additional subcellular compartmentalisation mechanism (Kelly *et al.*, 1998).

Preferential accumulation of the glucosylated form of scopoletin, designated scopolin, was found in tobacco plants upon challenge with Tobacco mosaic virus (TMV) or after treatment with the glycoprotein elicitor from *Phytophthora megasperma* (Costet *et al.*, 2002). Paradoxically, downregulation of the corresponding glucosyltransferase reduced the levels of both aglycone and glucoside at TMV infection foci (Chong *et al.*, 2002). Likewise, *Arabidopsis ugt71c1* glucosyltransferase mutants were found to contain reduced levels of both scopoletin and its glucoside (Kai *et al.*, 2006b). This might be indicative of a negative feedback mechanism in which the free aglycone directly or indirectly inhibits its own biosynthesis if the glucosylation step is malfunctioning.

8.4 Dynamic induction and targeting of secondary metabolites to the pathogen interface

The terminological distinction between phytoalexin-type and phytoanticipin-type metabolites suggests that only the former is integrated in active and dynamic plant responses to biotic stress cues while the latter compounds are considered to act as a passive antimicrobial barrier (VanEtten *et al.*, 1994). However, some examples suggest that mobilisation and activation of phytoanticipins from cellular stores can become integrated in dynamic plant responses to parasites.

8.4.1 Phytoalexin response

8.4.1.1 Induction

The concerted induction of phytoalexin accumulation with other plant defence responses after pathogen challenge suggests the existence of co-regulation and common 'upstream' inductive cues. A range of pathogen-derived structures known as general elicitors or pathogen-associated molecular patterns (PAMPs) as well as pathogen-derived toxins can trigger defence reactions, including phytoalexin accumulation. Classic examples are β-heptaglucan and Pep-13 oligopeptide that are derived from *Phytophthora sojae*. The former is a potent inducer of pterocarpan-type isoflavonoids in soybean (Sharp *et al.*, 1984), while the latter triggers accumulation of furanocoumarin phytoalexins in parsley cultured cells and leaves (Parker *et al.*, 1991). In *Arabidopsis*, induction of camalexin can be triggered by treatment of leaves with oomycete-derived Nep1-like proteins (Qutob *et al.*, 2006) or by

the *Fusarium*-derived phytotoxins, fusaric acid (Bouizgarne *et al.*, 2006) and fumonisin B1 (Stone *et al.*, 2000).

To date, only two PAMP receptors, *Arabidopsis* FLS2(flagellin sensing2) and EF-Tu receptor, have been identified, each composed of structural modules permitting perception of extracellular PAMPs and subsequent initiation of intracellular signalling. Both receptors contain extracellular leucine-rich repeats, a single transmembrane-spanning helix and an intracellular kinase domain. FLS2 directly binds to the flg22 peptide, the most conserved part of the bacterial motor protein flagellin, whereas EF-Tu receptor recognises elf18, an acetylated 18-mer peptide derived from the N-terminus of bacterial elongation factor EF-Tu (Gomez-Gomez and Boller, 2000; Zipfel *et al.*, 2006). Because both receptors contain an intracellular kinase module, signal transduction is likely initiated via phosphorylation cascades subsequent to binding of the cognate PAMPs to the extracellular leucine-rich repeats region. Although treatment of *Arabidopsis* plants with elf18 or flg22 or chitin changes the expression of a largely overlapping set of genes, suggesting convergence of signalling pathways triggered by different PAMP receptors (He *et al.*, 2007), still little is known about the exact signalling components and cascades relaying non-self-perception to the transcriptional activation of biosynthetic phytoalexin genes. A large number of *Arabidopsis* mutant lines are known to have defects in disease resistance responses of which many affect signalling components (Glazebrook, 2001). Some of these mutants point to links between the signalling molecule salicylic acid (SA) and phytoalexin induction. For example, *pad4* mutants that fail to accumulate the phytoalexin camalexin upon pathogen challenge (Glazebrook *et al.*, 1996) were later shown to have defects also in SA signalling, in basal defence responses to virulent pathogens as well as in race-specific *R* gene-triggered immunity (Zhou *et al.*, 1998). Although the exact biochemical function of PAD4 is still unclear, PAD4 forms a regulatory heterocomplex with a sequence-related protein, EDS1, and may act by amplifying EDS1-dependent defence upstream of SA (Feys *et al.*, 2001). Consistent with this, transgenic *Arabidopsis* lines expressing the bacterial NahG protein that degrades SA are strongly affected in camalexin accumulation upon challenge with virulent and avirulent *P. syringae* strains (Zhao and Last, 1996; Nawrath and Metraux, 1999). However, *sid2* mutant plants lacking isochorismate synthase, the principal enzyme used by *Arabidopsis* to synthesise SA from chorismate (Wildermuth *et al.*, 2001), showed increased camalexin levels compared to wild type (Nawrath and Metraux, 1999), indicating a more complex relationship between SA biosynthesis/signalling and camalexin accumulation.

8.4.1.2 Localisation

Knowledge of the tissue- and subcellular localisations of pathogen-induced metabolites is of critical importance to obtain insight into the dynamics of their mobilisation/activation and targeted accumulation. In simple terms, antimicrobials must be released at the right place and time in sufficient amounts

to fulfil defensive functions. Unfortunately, direct non-invasive imaging of phytochemicals with subcellular resolution is possible only for a small number of these molecules. Specific physicochemical properties can be exploited occasionally to visualise their accumulation using fluorescence microscopy techniques. Alternative approaches involve cell and/or organelle sorting coupled to subsequent liquid chromatography and/or mass spectrometry-based analysis. Ideally, combined spatio-temporal information should be available on the metabolite(s) and the activity of the corresponding biosynthetic genes.

High performance liquid chromatography analysis was used to examine the spatial distribution of camalexin adjacent to *Botrytis cinerea* spore inoculation sites in *Arabidopsis* leaves. Camalexin accumulated exclusively in the lesion zone and generated a gradient, declining from the lesion to background levels within a few millimetres (Kliebenstein *et al.*, 2005). Similarly, after inoculation with *A. alternata*, camalexin accumulation and the activation of camalexin biosynthetic genes including *ASA1*, *CYP79B2* and *PAD3* were restricted to sites of attempted infection (Schuhegger *et al.*, 2007). In cotton plants, sesquiterpenoid phytoalexins are induced by *X. campestris* pv. *malvacearum* challenge. Mesophyll cells undergoing a hypersensitive response (HR) adjacent to *X. campestris* microcolonies show fluorescence that is spectrally similar to one of the cotton phytoalexins (Pierce and Essenberg, 1987; Essenberg *et al.*, 1992). A fluorescence-activated cell sorter enabled the isolation of HR cells from inoculated cotyledons of a resistant cotton line and confirmed phytoalexin accumulation in these cells. The most abundant of these, 2,7-dihydroxycadalene, appears to accumulate in the fluorescent cells at concentrations that are about 40 times higher than in adjacent symptomless cells (Pierce and Essenberg, 1987).

One of the most spectacular examples illustrating targeted localisation of pathogen-triggered plant secondary metabolites at the subcellular level has been described in sorghum. Inoculation of sorghum plants with the maize pathogen *Colletotrichum graminicola* leads to the accumulation of red-coloured 3-deoxyanthocyanidin phytoalexins (Snyder and Nicholson, 1990). Microscopic studies revealed that these compounds accumulate in inclusion bodies that are formed in the cytoplasm of attacked epidermal cells. These vesicle-like structures focally accumulate beneath the fungal appressorium, leading to high local concentrations at pathogen entry sites (Fig. 8.7; Snyder and Nicholson, 1990; Nielsen *et al.*, 2004). The underlying mechanism might involve directed transport of intracellular vesicles along polarised actin cables, a common dynamic response of plant cells at pathogen contact sites (Kobayashi *et al.*, 1997; Takemoto *et al.*, 2003; Koh *et al.*, 2005). Vesicle trafficking in eukaryotic cells is typically controlled by soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins (see Chapter 7). In this context, it is noteworthy that two functionally homologous syntaxins (t-SNAREs), *Arabidopsis* PEN1 and barley ROR2, focally accumulate in lipid raft-like plasma membrane microdomains directly beneath attempted fungal entry sites (Assaad *et al.*, 2004; Bhat *et al.*, 2005). Since loss-of-function

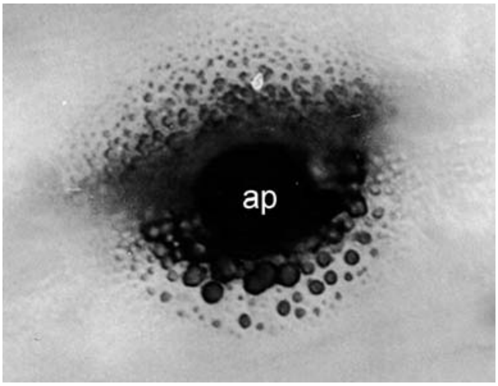


Figure 8.7 Vesicle-like structures containing pigmented 3-deoxyanthocyanidin phytoalexins accumulates at the site of attachment of a *Colletotrichum graminicola* appressorium in a resistant sorghum line. Visualised by light microscopy. ap, appressorium. (Reproduced from Morrissey and Osbourn (1986), by permission of The American Society for Microbiology.)

mutations in the corresponding genes impair disease resistance responses, leading to fungal entry, this demonstrates a resistance function for SNARE proteins. Recently, *Arabidopsis* PEN1 was shown to form ternary SNARE complexes with the SNAP33 adaptor and a subset of vesicle-associated membrane proteins (VAMPs), including VAMP721 and VAMP722 (Kwon *et al.*, 2008). Since the latter two proteins are essential to restrict fungal entry and because GFP-VAMP722 marked mobile vesicles congregate at fungal entry sites, this strongly suggests that SNARE-mediated vesicle fusion at the plasma membrane (i.e. exocytosis) is critical for disease resistance responses. However, it remains unclear whether *Arabidopsis* VAMP721 and VAMP722 vesicles transport antimicrobial compounds and/or enzymes involved in their biosynthesis.

8.4.2 Waking up sleeping dogs: phytoanticipin activation

8.4.2.1 Localisation of preformed antimicrobial metabolites

Fluorescent properties of avenacins A-1 and B-1 that are attributed to the anthranilic acid residue present in the aglycone backbone enabled the localisation of these compounds almost exclusively in vacuoles of root epidermal cells of *Avena sativa* and *Avena strigosa* (Osbourn *et al.*, 1994; Field *et al.*, 2006). Since epidermal cells are typically the first contact sites of a microbial parasite and because many fungal pathogens colonise host tissue by invasion/penetration of epidermal cells, the exquisite tissue-specific accumulation pattern of avenacins is consistent with their proposed protective function. In leaves, both epidermal and mesophyll cells contain avenacosides. However, leaf

epidermal tissue contains almost exclusively avenacoside B, whilst mesophyll cells contain both avenacosides A and B (Kesselmeier and Urban, 1983).

Quantitative analysis of benzoxazinone glucoside levels in various maize and wheat tissues revealed that their concentration is usually highest in vascular tissue of leaves and roots (Argandona and Corcuera, 1985; Argandona *et al.*, 1987). In wheat, the lower leaf epidermis appears to lack benzoxazinone (Argandona *et al.*, 1987). Accumulation of benzoxazinones in vascular tissue correlates with the observation that differential *H. turcicum* growth between resistant and susceptible maize lines was detectable when the fungal mycelium reached vascular bundles (Hilu and Hooker, 1964; Mace, 1973).

8.4.2.2 Activation of phytoanticipin precursors

In essentially all cases where activation of a phytoanticipin is dependent on the cleavage of a glycosidic bond by a corresponding hydrolytic enzyme, pathogen-mediated host tissue damage is thought to bring the glycosyl hydrolase in physical contact with its substrate (Osbourn, 1996; Sicker *et al.*, 2000; Brader *et al.*, 2001). If this paradigm is correct, then the release of toxic principles during plant pathogen encounters would occur only at later stages of pathogenesis coincident with cellular disintegration of host tissue. In addition, this mode of activation is expected to work best against microbial invaders if enzyme substrate pairs occur in the same cell and are separated from each other at the subcellular level (as shown for bisdesmosidic saponins or benzoxazinone glucosides). However, if enzyme substrate pairs are compartmentalised in different cell types as proposed for glucosinolates (Koroleva *et al.*, 2000; Andreasson *et al.*, 2001), the release of biologically active end products does require tissue destruction and mixing of different cell-type contents. Such massive tissue damage typically results from insect feeding, but is normally not seen at early stages of microbial pathogenesis.

Rapid dynamic relocation of enzymes and/or substrates in intact cells upon pathogen attack could serve as an alternative, more subtle phytoanticipin activation mechanism. Such relocation events might occur within a single cell or between cells. A dynamic activation mode of toxic principles would be advantageous since it could target an invader at early stages of pathogenesis, i.e. prior to host cell entry and/or tissue destruction. This would enable plants to concentrate active molecules to relatively high levels at the plant–microbe interface (Fig. 8.8). The peroxisome-associated predicted PEN2 glucosylhydrolase (Lipka *et al.*, 2005) might serve as an example of a dynamic relocalisation-dependent activation mechanism for antimicrobials. Upon establishment of fungal contact sites on the *Arabidopsis* leaf surface and invasion attempts into single leaf epidermal cells, PEN2-associated peroxisomes focally accumulate beneath attempted entry sites (Fig. 8.9). Lack of PEN2 permits effective entry of several tested fungal parasites into epidermal cells (Lipka *et al.*, 2005). Dynamic relocalisation and the congregation of peroxisomes is likely the consequence of a rapid polarisation of the host actin cytoskeleton to pathogen contact sites because peroxisomes move exclusively along actin filaments,

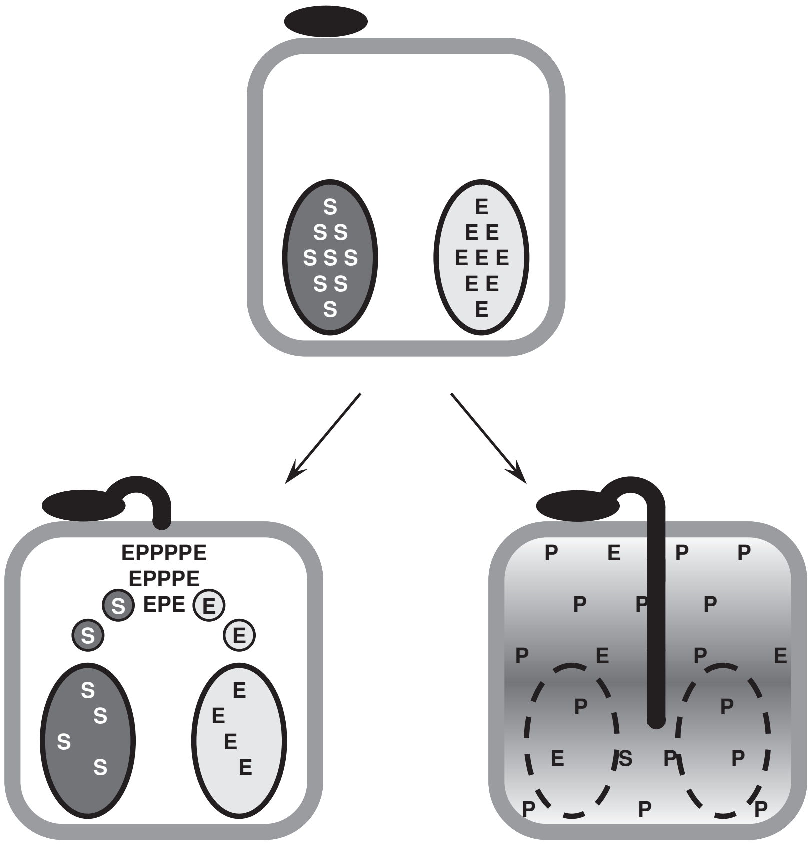


Figure 8.8 Two possible models for activation of constitutive phytochemicals. The precursor molecules and respective activating enzymes are constitutively separated on the subcellular level (upper panel). Shown are the dynamic model (left panel) and the passive model of activation (right panel). E, enzyme; S, substrate; P, toxic product.

and treatment of plant tissue with actin cytoskeleton inhibitors or genetic perturbation of actin dynamics produces a *pen2* phenocopy (Kobayashi *et al.*, 1997; Miklis *et al.*, 2007). Since it is likely that PEN2 activates yet unidentified glucose conjugate(s) of *Arabidopsis* secondary products, the enzyme might act on substrate(s) only after its pathogen-triggered relocalisation, thereby releasing at high concentrations biologically active aglycone(s) at attempted entry sites.

8.4.3 Secretion

Unlike many fungal pathogens, all examined bacterial pathogens of plants remain outside plant cells. This and the observation that pathogenesis of many potential fungal or oomycete parasites is frequently terminated prior to

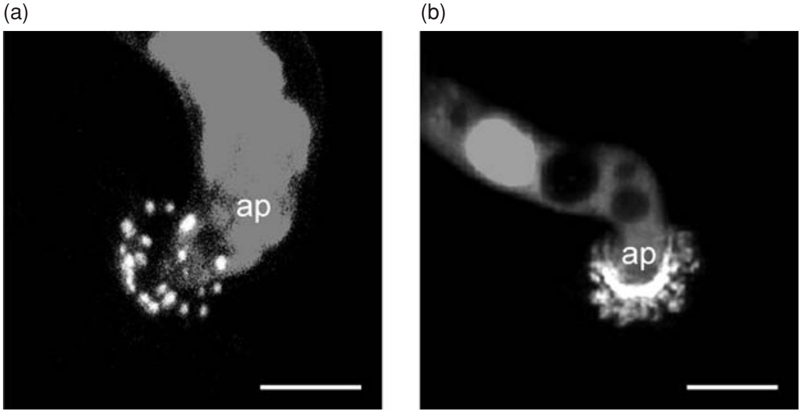


Figure 8.9 Peroxisomes containing PEN2-GFP accumulate at *Blumeria graminis* entry sites in an *Arabidopsis* leaf epidermal cell (a) 16 h and (b) 30 h after inoculation. Visualised by confocal laser scanning microscopy. Fungal structures are stained by propidium iodide. Scale bar, 10 μm. ap, appressorium. (Courtesy of Riyaz Bhat.)

invasive growth point to the importance of active resistance mechanisms acting outside of cells. In this context, the pathogen-inducible secretion of plant secondary products could have general significance for growth restriction of diverse pathogen classes. Many metabolites, including antimicrobials, are known to be secreted by roots into the rhizosphere. This is important for symbiotic as well as parasitic plant–microbe interactions (Bais *et al.*, 2006). Root exudates can contain constitutive as well as inducible phytochemicals. For example, maize roots secrete benzoxazinone aglycones (Zhang *et al.*, 2000). Likewise, *Arabidopsis* roots exude a range of phenolic- as well as indolic-type compounds upon inoculation of non-adapted *P. syringae* or *P. sylvaticum* isolates (Bais *et al.*, 2005; Bednarek *et al.*, 2005). The pathogen-triggered secretion of metabolites is not restricted to plant roots, as some compounds were also detected on the leaf surface. Phytoalexins can be detected in inoculation droplets a few hours after spore inoculations of the leaf surface. Examples of this are scopoletin secretion by rubber tree leaves after challenge with *Phytophthora palmivora* (Churngchow and Rattarasarn, 2001) or camalexin secretion by *Arabidopsis* leaves upon *Cochliobolus carbonum* droplet application (Zook and Hammerschmidt, 1997).

Despite its apparent significance, little is known about the molecular mechanisms underlying the secretion of antimicrobials. The *Np*ABC1 ATP-binding cassette-type (ABC) transporter from *Nicotiana plumbaginifolia* resides in the plasma membrane and is thought to be involved in the secretion of sclareol, an antifungal terpenoid on the leaf surface of *Nicotiana* spp. (Bailey *et al.*, 1975; Jasinski *et al.*, 2001). This was based on *NpABC1* gene activation and accumulation of NpABC1 in the plasma membrane following application of sclareolide or sclareol to suspension-cultured tobacco cells and concomitant

secretion of a labelled synthetic sclareolide derivative (Jasinski *et al.*, 2001). Another ABC transporter that could be involved in the secretion of plant antimicrobials is *Arabidopsis* PEN3/PDR8 (Stein *et al.*, 2006). This plasma membrane-resident transporter focally accumulates beneath sites of attempted fungal entry of leaf epidermal cells. Mutant *pen3* plants permit increased entry rates of the non-adapted powdery mildews *Blumeria graminis* or *Erysiphe pisi* and are hypersusceptible to the necrotrophic fungus *Plectosphaerella cucumerina* (Stein *et al.*, 2006). This suggests that PEN3 serves as a component of a machinery for targeted secretion of antimicrobials and/or chemical cross-linking of the cell wall. Since PEN3 acts together with PEN2 in a common genetic pathway (Stein *et al.*, 2006), it is possible that PEN3 translocates PEN2 glycosyl hydrolase-activated molecules into the apoplastic space.

A number of plant secondary products, mainly of phenolic type, are covalently bound to cell wall components. Many representatives of this subgroup are pathogen-inducible and are therefore undetectable in cell wall fractions of healthy plant tissue (McLusky *et al.*, 1999; Hagemeier *et al.*, 2001; Tan *et al.*, 2004). Thus, these cell wall-bound compounds must be translocated through the plasma membrane before they reach their destination compartment, a process requiring active transport mechanisms. Of note is a report of directed transport of fluorescent phenolics in vesicle-like structures in onion epidermal cells at attempted entry sites of the necrotrophic fungus *Botrytis allii*. The polarised transport correlates with a chemical cell wall reinforcement that involves binding by ether linkages of autofluorescent hydroxycinnamic acid amides feruloyl-3′-methoxytyramine and feruloyltyramine (McLusky *et al.*, 1999). One possibility is that a common vesicle-based secretion system permits the transport and targeted delivery of chemically diverse compounds in different plant species, such as hydroxycinnamic acid amides in onion and red-coloured 3-deoxyanthocyanidin phytoalexins in sorghum (see above). However, sclareol secretion by the plasma membrane-resident ABC transporter *Np*ABC1 in *N. plumbaginifolia* as well as *Arabidopsis* PEN3 ABC transporter-dependent resistance to fungal entry (see above) indicates that plant cells engage at least two delivery mechanisms for the release of small molecules on the cell surface.

8.5 Genetic approaches to validate defensive functions of plant secondary metabolites

Unambiguous functional assignment of a secondary metabolite biosynthetic pathway and its corresponding products to plant disease resistance responses is a major challenge. One of the reasons for this is that pathogens evolved strategies to detoxify antimicrobials (see below). In addition, microbial parasites invented various entry and colonisation mechanisms to avoid the release of or direct contact with toxic compounds. Finally, microbial pathogens

evolved a battery of effectors that are delivered into plant cells to suppress inducible plant immune responses at attempted colonisation sites. This typically involves interception of signalling steps triggered upon non-self-perception of PAMPs by surface resident pattern recognition receptors (PRRs; Jones and Dangl, 2006; He *et al.*, 2007).

8.5.1 Natural genetic variation

Genetic methods are essential to establish functional links between a particular group of secondary metabolites and their physiological significance in restricting pathogen entry and/or colonisation. One approach utilises natural variation of secondary metabolites among closely related plant species or among accessions within a species to correlate the presence and amount of a particular metabolite with infection phenotypes upon inoculation with a particular pathogen. A survey of different oat species led to the identification of a diploid species, *Avena longiglumis*, which fails to accumulate avenacin. This species was found to be susceptible to the avenacin sensitive fungus *G. graminis* var. *tritici* that fails to colonise other avenacin-containing oats (Osbourn *et al.*, 1994). However, as *A. longiglumis* does not hybridise easily with any other diploid oat species, it could not be tested whether the ability to synthesise avenacins and disease resistance to *G. graminis* var. *tritici* are genetically linked.

Similarly, the presumed role of glucosinolates for disease resistance was approached by classical breeding experiments. A total of 33 *B. napus* lines varying both in the total content and in composition of these metabolites were tested with *Leptosphaeria maculans* and *Alternaria* spp. under field conditions. In this case, alterations of glucosinolate accumulation in leaves did not affect resistance of *B. napus* to *L. maculans*, while for *Alternaria* higher glucosinolate content appeared to correlate with enhanced disease susceptibility (Giamoustaris and Mithen, 1997), thereby refuting a simple positive correlation between glucosinolate content and disease resistance.

8.5.2 The power and pitfalls of mutational studies of secondary metabolite pathways

In cases where a positive correlation between the presence of a potential antimicrobial and disease resistance to a particular pathogen has been shown, loss-of-function mutations in genes required for the synthesis of a secondary metabolite can deliver clear evidence for in vivo function(s) of metabolites. However, this approach is also not without potential misinterpretations. For example, mutations in regulatory genes of a secondary biosynthetic pathway or in broadly effective defence signalling genes might impair several unrelated pathways/processes, thereby complicating the interpretation of altered infection phenotypes (see below). Whilst genetic redundancy of biosynthetic steps can be normally overcome by the generation of double mutants, a

genetic lesion in a biosynthetic pathway can occasionally lead to unexpected compensatory changes in related biosynthetic pathways. This complication is likely a consequence of built-in connection nodes between metabolic pathways.

A good example is the *Arabidopsis* glucosinolate pathway, one of the best characterised plant secondary metabolite biosynthetic systems (Grubb and Abel, 2006; Halkier and Gershenzon, 2006). Both genetic redundancy and an inherent biosynthetic link between indole glucosinolates and indole-3-acetic acid obstruct the isolation of mutants specifically lacking glucosinolates. The only biosynthetic mutant reported so far is *sur1*, which accumulates extremely high auxin levels leading to severe growth anomalies and lethality at later stages of development (Mikkelsen *et al.*, 2004). This makes *sur1* plants almost useless for meaningful infection experiments with pathogens.

An example that specifically links the function of metabolic pathway genes to disease resistance by gene disruption experiments has been described for the benzoxazinone pathway in maize. Recessive *bx1* mutations severely reduce DIMBOA biosynthesis in *bx1* homozygous mutant plants by eight- to tenfold in comparison to wild type (Hamilton, 1964). The mutant plants are extremely susceptible to *H. turcicum*, a causal agent of northern corn leaf blight (Couture *et al.*, 1971). Cloning of the *BX1* gene and subsequent biochemical characterisation of the encoded protein revealed an enzymatic activity in the biosynthesis of benzoxazinone (Frey *et al.*, 1997). This finding supported earlier conclusions of Couture *et al.* (1971) that the enhanced susceptibility of *bx1* plants results from strongly reduced DIMBOA levels.

A mutational approach was also used to dissect in vivo functions of saponins. Chemical mutagenesis of diploid *A. strigosa* enabled the isolation of saponin-deficient (*sad*) mutants defining at least seven genetic loci (Papadopoulou *et al.*, 1999; Haralampidis *et al.*, 2001). Since the *sad* mutants show wild-type levels of closely related avenacosides, it is likely that the corresponding wild-type genes specifically impair biosynthetic or regulatory steps in the biosynthesis of avenacins (Papadopoulou *et al.*, 1999). Indeed, *SAD1* and *SAD2* encode enzymes that are directly involved in avenacin biosynthesis (Haralampidis *et al.*, 2001; Qi *et al.*, 2006). Homozygous *sad* mutant lines were used in pathogen assays to test the presumed role of avenacins in disease resistance. This revealed enhanced disease susceptibility to the soilborne wheat fungus *G. graminis* var. *tritici*, which usually does not infect oats. Moreover, the mutants were hypersusceptible to the adapted strain *G. graminis* var. *avenae* as well as to *F. culmorum* and *Fusarium avenaceum* (Papadopoulou *et al.*, 1999). Since the mutations did not alter infection phenotypes of leaf-infecting *Stagonospora* species in both host and non-host interactions, this supports specific defects of the *sad* mutants in root-restricted avenacine accumulation (Papadopoulou *et al.*, 1999).

Arabidopsis is an excellent model to study phytoalexin functions (Glawischnig, 2007). A series of five non-allelic phytoalexin deficient (*pad*) mutants with defects in the accumulation of pathogen-inducible camalexin were

isolated from an ethylmethanesulfonate-mutagenised population (Glazebrook and Ausubel, 1994; Glazebrook *et al.*, 1996, 1997). However, initial pathogen assays of *pad* mutants with virulent and avirulent strains of *P. syringae* produced seemingly contradictory data regarding a presumed camalexin function in bacterial growth restriction. Although some *pad* lines showed enhanced susceptibility to virulent strains of *P. syringae*, there was no simple correlation between phytoalexin levels and disease resistance. Especially, *pad3* lines with undetectable camalexin levels gave wild-type-like susceptibility in both compatible and incompatible *P. syringae* interactions (Glazebrook and Ausubel, 1994). This led to two major conclusions: (1) camalexin is not of functional significance in restricting growth of *P. syringae* in *Arabidopsis* and (2) only a subset of *PAD* genes encode enzymes involved in camalexin biosynthesis while others have regulatory functions in multiple defence pathways. This inference was confirmed by the isolation and characterisation of *PAD4*, encoding a lipase-like protein, that is engaged in diverse disease resistance responses, including SA-dependent signalling (Zhou *et al.*, 1998; Jirage *et al.*, 1999). Mutant lines *pad3* and *pad5*, which retained full resistance to *P. syringae*, were predicted to carry mutations in genes encoding proteins that are directly involved in camalexin biosynthesis. This was proven for *PAD3* encoding a putative P450 monooxygenase (designated CYP71B15; Zhou *et al.*, 1999), whilst the isolation of *PAD5* has not been reported to date. Recently, PAD3 was shown to catalyse the last step in camalexin biosynthesis, the conversion of camalexic acid to camalexin (Schuhegger *et al.*, 2006). This finding permitted the use of *pad3* as excellent tool for probing specific camalexin functions in disease resistance to other microbial pathogens. Indeed, *pad3* plants exhibit enhanced disease susceptibility to necrotrophic fungi including *Alternaria brassicicola* (Thomma *et al.*, 1999) and some *B. cinerea* isolates (Ferrari *et al.*, 2003; Kliebenstein *et al.*, 2005).

Since the early reports on the isolation of *pad* mutants, additional lines affected in camalexin accumulation have been reported (Glawischnig *et al.*, 2004; Denby *et al.*, 2005; Nafisi *et al.*, 2007). Similar to *pad1*, *pad2* and *pad4*, the *ups1* mutant seems to be affected in a regulatory rather than biosynthetic component (Denby *et al.*, 2005). The *cyp71A13* biosynthetic knockout, permitting reduced levels of camalexin accumulation after pathogen treatment, shows enhanced disease susceptibility to *A. brassicicola* but not to *P. syringae* (Nafisi *et al.*, 2007). This confirms earlier conclusions on camalexin function based on *pad3* infection phenotypes.

The *Arabidopsis cyp79B2 cyp79B3* double mutant is an interesting biosynthetic mutant since it compromises both camalexin biosynthesis (Glawischnig *et al.*, 2004) and the accumulation of other tryptophan-derived metabolites, including indole glucosinolates (Zhao *et al.*, 2002) and derivatives of indole-3-carboxylic acid (P. Bednarek and P. Schulze-Lefert, unpublished data). Importantly, accumulation of the tryptophan-derived plant hormone indole-3-acetic acid in *cyp79B2 cyp79B3* plants is affected very weakly, if at all

(Zhao *et al.*, 2002), suggesting specialised functions of CYP79B2 and CYP79B3 enzymes in the biosynthesis of tryptophan-derived secondary metabolites. Interestingly, the double mutant exhibited a greater level of susceptibility to some isolates of *B. cinerea* than *pad3* plants (Kliebenstein *et al.*, 2005). Moreover, *cyp79B2 cyp79B3* plants are, compared to wild type, super-susceptible to several tested powdery mildew fungi, including non-adapted *B. graminis* and *E. pisi* as well as adapted *Golovinomyces orontii* (P. Bednarek and P. Schulze-Lefert, unpublished data). Since *pad3* plants do not permit enhanced powdery mildew growth, this suggests that tryptophan-derived secondary metabolites other than camalexin contribute to growth restriction of necrotrophic and biotrophic fungal pathogens. However, the fact that multiple groups of indole-type secondary products fail to accumulate in *cyp79B2 cyp79B3* plants makes it difficult to assign a functional contribution of individual compounds or compound groups to the observed infection phenotypes. Candidate compounds are indole glucosinolates and their degradation products, indole-3-carboxylic acid derivatives, any unidentified metabolites derived from indole-3-acetaldoxime, the substrate of CYP79B2 and CYP79B3 enzymes, or combinations of these metabolites. Future functional dissection of single tryptophan-derived metabolites or their subclasses in *Arabidopsis* resistance responses requires the availability of mutants with defects in particular branches of tryptophan metabolism. Unfortunately, such mutants have not been reported so far. Of note, the *cyp79B2 cyp79B3* knockout line permits similar *P. syringae* growth as wild type (Nafisi *et al.*, 2007), suggesting either that all presently known tryptophan-derived metabolites are irrelevant for bacterial growth restriction or that the bacterium can effectively detoxify and/or suppress accumulation of these compounds at colonisation sites.

8.5.3 Metabolite engineering and plant disease resistance

Genetically modified plants can deliver crucial information about functions of phytochemicals in plant defence. This approach is often referred to as metabolite engineering. Transgenesis of model plants is aimed to either modify accumulation levels of native plant metabolites or introduce novel antimicrobial compounds. Such experiments can assist in clarifying the physiological function(s) of native phytochemicals in disease resistance and might reveal whether a novel metabolite alters the range of pathogens that parasitise a particular plant species.

Constitutive overexpression of the alfalfa isoflavone *O*-methyltransferase gene in alfalfa resulted in a more rapid and increased production of the pterocarpan phytoalexin medicarpin. In addition, transgenic lines display resistance to the fungus *Phoma medicaginis*, supporting the functional significance of medicarpin in fungal growth restriction in this interaction (He and Dixon, 2000). The contribution of scopoletin accumulation to viral disease resistance was tested by both overexpression as well as downregulation of

UDP-Glc:phenylpropanoid glucosyltransferases that act efficiently on the hydroxycoumarin scopoletin and on hydroxycinnamic acids. Glucosyltransferase-depleted lines revealed both reduced accumulation of scopolin and scopoletin and higher susceptibility to the TMV virus (Chong *et al.*, 2002). In contrast, overexpression of the respective glucosyltransferase led to higher levels of both metabolites after virus inoculation and a faster HR execution, but not to elevated TMV resistance (Gachon *et al.*, 2004). However, the overexpressor lines appear to be more resistant to *Potato virus Y* (Matros and Mock, 2004). Unless scopoletin/scopolin null mutants become available, we cannot exclude the possibility that both downregulation as well as overexpression of the glucosyltransferase genes had additional effects on infection phenotypes of the transgenic plants that are unrelated to scopoletin/scopolin accumulation.

Putative functions of glucosinolates in plant defence responses were tested by exposing *Arabidopsis* transgenic lines producing novel glucosinolate structure types to pathogens (Brader *et al.*, 2006). Since *Arabidopsis* naturally produces glucosinolates, the metabolite engineering was in this case aimed at widening the spectrum of glucosinolate structure types in a species by expression of P450 monooxygenases (CYPs) that convert different amino acids to their respective oximes. The oximes are subsequently channelled into the glucosinolate pathway due to the relative low amino acid specificity of post-aldoxime enzymes in glucosinolate biosynthesis (Grubb and Abel, 2006). Transgenic *Arabidopsis* lines were generated expressing either the cassava valine converting CYP79D2 or sorghum tyrosine converting CYP79A1 or overexpressing endogenous phenylalanine converting CYP79A2. Infection phenotypes of these lines were tested with *A. brassicicola*, *E. carotovora* subsp. *carotovora* and *P. syringae* pv. *tomato* DC3000. Expression of *CYP79A1* and *CYP79A2* led to slightly enhanced resistance to *P. syringae* but higher susceptibility to *A. brassicicola.* Plants expressing *CYP71D2* were more resistant to *E. carotovora* but responded in the same way as wild type to *P. syringae* or *A. brassicicola* (Brader *et al.*, 2006). However, altered infection phenotypes of *CYP79A1* and *CYP79A2* expressing plants were attributed to spurious induction of SA and suppression of jasmonate-dependent defence signalling rather than a direct antimicrobial activity of glucosinolates or their degradation products (Brader *et al.*, 2006). Possibly, only the enhanced disease resistance phenotype of *CYP71D2* lines to *E. carotovora* is due to direct effects of novel glucosinolate types.

Although gene transfer techniques can, in principle, permit the entire transfer of a metabolic pathway from one species into another, thereby introducing completely novel metabolic product classes, this is difficult to achieve due to complex regulatory connectivities between different endogenous metabolic pathways. One exception from this rule is the biosynthesis of stilbene-type phytoalexins with resveratrol as major representative. The biosynthesis of the core stilbene structure requires the presence of one enzyme only – STS (Tropf *et al.*, 1994). In addition, the precursor molecules required for

stilbene biosynthesis, *p*-coumaroyl-CoA and malonyl-CoA, are constitutively synthesised by plants (Dixon and Paiva, 1995), which makes this compound an ideal candidate for metabolic engineering. The grapevine *STS* gene *vst1* was expressed under the control of its own pathogen-inducible promoter in tobacco (Hain *et al.*, 1993). The transgenic lines showed pathogen-inducible accumulation of *STS* mRNA, of resveratrol, and displayed enhanced disease resistance to *B. cinerea*. The degree of resistance between individual lines correlated with the kinetics of *STS* mRNA induction and with the concentrations of resveratrol in leaf tissue (Hain *et al.*, 1993). Likewise, expression of *STS* under its native promoter in rice increased disease resistance to *Pyricularia oryzae* (StarkLorenzen *et al.*, 1997) and in tomato to *P. infestans* (Thomzik *et al.*, 1997). The transgenic tomato plants exhibited wild-type levels of resistance against *B. cinerea* and *Alternaria solani*, although resveratrol accumulated to the same levels as during interactions with *P. infestans*.

When the peanut *STS* cDNA was constitutively expressed in transgenic alfalfa by the *Cauliflower mosaic virus* 35S promoter, resveratrol glucoside accumulated and this correlated with enhanced disease resistance to *Phoma medicaginis* (Hipskind and Paiva, 2000). In vitro tests showed that both resveratrol and resveratrol glucoside greatly inhibit hyphal growth of *P. medicaginis* (Hipskind and Paiva, 2000). These findings illustrate that a change in the expression pattern of a single metabolic gene can convert a phytoalexin into a phytoanticipin. This study also demonstrates that in a heterologous species a novel metabolite can be metabolised further by native host plant enzymes: resveratrol is converted to resveratrol glucoside by an endogenous alfalfa glucosyltransferase.

8.6 Successful pathogens

8.6.1 Attenuation of plant defence responses

One common strategy of virulent pathogens involves the deployment of defence suppression mechanisms. By this means, either host recognition is avoided or PRR-triggered disease resistance responses are intercepted by pathogen-derived effector molecules (Jones and Dangl, 2006; He *et al.*, 2007). Since characterised pathogen effectors appear to target early signalling steps of PRR-triggered disease resistance responses, this is expected to either delay or block the induction/activation of all ‘downstream’ executors including phytoalexins and phytoanticipins. Indeed, phytoalexins were found to accumulate earlier and to higher levels in *R* gene-triggered incompatible interactions than in compatible interactions (Hammerschmidt, 1999). Accumulation of *Arabidopsis* camalexin and derivatives of indole-3-carboxylic acid is induced faster and reaches higher levels upon challenge with *P. syringae* pv. *tomato* DC3000 expressing the avirulence gene *avrRpm1* compared to the

virulent strain lacking this effector (Hagemeier *et al.*, 2001; Tan *et al.*, 2004). Of note, *P. syringae* pv. *tomato* DC3000 is able to suppress root secretion of antimicrobial compounds, infect roots and cause disease, whereas *hrp* mutants of this bacterial strain (*hrcC* or *hrpL*) trigger exudation of antimicrobials and fail to colonise roots (Bais *et al.*, 2005). Since the bacterial type III (Hrp) secretory system is essential for translocation of a diverse set of bacterial effectors into plant cells, the effectors likely intercept disease resistance signalling upstream of the biosynthesis and/or secretion of antimicrobials.

8.6.2 Counterdefence: how pathogens overcome antimicrobial compounds

Adapted pathogens are often more sensitive in vitro to host-derived antimicrobials than non-adapted pathogens. For example, oomycete pathogens like *Pythium* or *Peronospora* species are largely insensitive to saponins (Arneson and Durbin, 1968). This was attributed to an exceptionally low sterol content of their plasma membranes. Consistent with this, the in vitro sensitivity of *P. megasperma* to α-tomatine positively correlates with increasing concentrations of sterols supplied in the growth medium (Steel and Drysdale, 1988).

Evidence for the existence of specific detoxification mechanisms for avenacins came from the early observation that the causal agent of the 'take-all' disease in oat *G. graminis* var. *avenae* shows in vitro greater resistance to avenacins than the closely related non-adapted *G. graminis* var. *tritici* (Maizel *et al.*, 1964; Crombie and Crombie, 1986). Indeed, *G. graminis* var. *avenae*, but not var. *triciti*, as well as *Fusarium avenaceum* and *Stagonospora avenae* f.sp. *avenaria* evolved enzymes that detoxify these phytoanticipins by cleaving one of the glycosidic bonds in the branched oligosaccharide chain attached at the C-3 position of the aglycones (Crombie *et al.*, 1986b; Hughes *et al.*, 2004). Recall that the presence of an intact sugar residue at this position is essential for membranolytic activity of saponins (Keukens *et al.*, 1995; Armah *et al.*, 1999). Similarly, some adapted pathogens of tomato are less sensitive in vitro to α-tomatine than non-adapted pathogens (Arneson and Durbin, 1968). This was attributed to their ability to detoxify α-tomatine by specific glycosidases known as tomatinases. These enzymes cleave sugar residues from the oligosaccharide at the C-3 position of α-tomatine and are believed to be crucial for pathogenesis of *Septoria lycopersici* and *Fusarium oxysporum* f.sp. *lycopersici* (Lairini *et al.*, 1996; Melton *et al.*, 1998). Consistent with this, heterologous expression of a tomatinase in *Cladosporium fulvum*, a tomatine-sensitive tomato pathogen, showed increased sporulation on cotyledons of susceptible tomato lines and caused more extensive infection of seedlings of resistant tomato lines (Melton *et al.*, 1998).

Some phytopathogenic fungi can metabolise benzoxazolinones, which have antimicrobial activity in vitro and can form spontaneously from benzoxazinone aglycones (Oikawa *et al.*, 2004). The endophytic maize pathogen *F. moniliforme* is capable of catabolising both BOA and MBOA (Richardson

and Bacon, 1995). Interestingly, a rice isolate of this pathogen, a non-benzoxazinone-producing species, is unable to metabolise these two compounds. However, since this isolate was still able to infect maize plants, it is unlikely that benzoxazolinone degradation has a direct role for fungal virulence (Richardson and Bacon, 1995). Some *G. graminis* isolates can also degrade BOA and MBOA (Friebe *et al.*, 1998). In this case, *G. graminis* var. *graminis* catabolised both metabolites more efficiently than *G. graminis* var. *avenae* and *G. graminis* var. *triciti* (Friebe *et al.*, 1998). The ability of these *Gaeumannomyces* isolates to cause root rot symptoms in wheat parallels their potential to degrade the wheat-derived benzoxazolinones to non-toxic compounds.

Camalexin, the *Arabidopsis* phytoalexin, was shown to be metabolised to 5-hydroxycamalexin by the root pathogen *Rhizoctonia solani* (Conn *et al.*, 1994), while *Sclerotonia sclerotiorum* detoxifies camalexin by glucosylation (Pedras and Ahiahonu, 2002). Similarly, virulent pea isolates of *Nectria haematococca* detoxify the phytoalexin pisatin by demethylation (Ciuffetti and VanEtten, 1996; Wasmann and VanEtten, 1996).

8.6.3 Functional significance of pathogen resistance to toxic plant metabolites

Genetic approaches in microbial pathogens are of critical importance to obtain deeper insights into microbial detoxification mechanisms and their presumed role in virulence. The isolation of α-tomatine-resistant *Fusarium solani* mutants revealed that these not only were more resistant to α–tomatine in vitro, but were also able to infect green tomato fruits that contain high levels of α-tomatine and are usually not infected by *F. solani* (Defago *et al.*, 1983; Defago and Kern, 1983). Notably, the mutants accumulated reduced levels of sterols, supporting the idea that lipid membrane composition is a critical determinant of saponin resistance in fungi.

The functional significance of avenacinases and tomatinases was tested using targeted gene disruption techniques. A *G. graminis* var. *avenae* avenacinase mutant lacked avenacinase activity and was about eight times more sensitive in vitro to avenacin A-1. Importantly, the fungal mutant was unable to colonise root epidermal tissue whilst retaining its pathogenicity on wheat (Bowyer *et al.*, 1995). A similar approach was used to test the in vivo function of tomatinase from *S. lycopersici*. Remarkably, this revealed a dual role of tomatinase in fungal pathogenesis: the tomatinase gene is essential for saponin detoxification and contributes to the suppression of basal plant defence responses (Bouarab *et al.*, 2002). The exact mechanism of the latter function is not clear, but might be mediated by the product of tomatinase activity.

A number of camalexin-resistant mutants of the bacterial pathogen *P. syringae* pv. *maculicola*, which is virulent on *Arabidopsis*, were isolated (Rogers *et al.*, 1996). Although these mutants were not fully insensitive to the phytoalexin, they tolerated in vitro higher concentrations of camalexin than the wild-type strain. Since the bacterial growth curves of wild-type and mutant

P. syringae on *Arabidopsis* were indistinguishable, this strongly argues against a role of camalexin in restricting the in vivo growth of this particular pathogen.

Isolation of the pisatin demethylase (*PDA*) gene from *N. haematococca* provided an opportunity to assess the functional significance of pisatin detoxification for virulence. Targeted disruption of *PDA* (Wasmann and VanEtten, 1996) or transformation of a *N. haematococca pda* strain with *PDA* (Ciuffetti and VanEtten, 1996) resulted only in a minor decrease or increase, respectively, in pathogen virulence. These observations point to a minor role of pisatin detoxification for *N. haematococca* pathogenicity on pea.

8.7 Concluding remarks

The role of secondary metabolites in plant immune responses is now firmly established since their original discovery more than 60 years ago. Although the biosynthesis and accumulation of small antimicrobial molecules is one of the evolutionarily conserved responses of flowering plants to pathogenic microorganisms, we are only now beginning to understand why it is so difficult to unambiguously assign a defensive function to a particular compound: millions of years of co-evolution of plant–microbe interactions have left genetic footprints in both organisms that are the result of reiterated defence and counter-defence cycles. Because of this, only in few cases is a single phytochemical expected to terminate microbial pathogenesis. Thus, the physiological significance of antimicrobials in plant defence can be better valued if the co-evolutionary history of host and parasite is known. Novel DNA sequencing technologies are currently revolutionising the sequencing of complete eukaryotic genomes, and we predict that leads for generating natural chemical diversity in plants as well as corresponding detoxification mechanisms in pathogens will come from evolutionary genomics approaches.

The exact mechanisms underlying the dynamic induction and relocation of secondary metabolites at both inter- and intracellular levels remain one of the major future challenges. Integrated approaches including genetics, cell biology and biochemistry are needed to unravel the machinery permitting targeted delivery and secretion of antimicrobials at the host–microbe interface. We expect a limited number of cellular mechanisms for the transport of diverse biosynthetic enzymes and enzymatic products. Knowledge in this area is likely driven by a better understanding of the plant endomembrane system.

Finally, a full appreciation of plant secondary metabolite pathways in the plant immune system demands knowledge about their connectivity to 'upstream' signalling proteins as well as to other prominent immune response effector families, including defence-related proteins (van Loon *et al.*, 2006) or proteins altering cellular redox state. Knowledge on such regulatory links will be critical to predict the evolvability of secondary metabolite pathways in a particular plant species. This is expected to reveal constraints and

provide opportunities for future rationale modification of secondary metabolite pathways towards improved disease resistance.

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Chapter 9

CHEMICAL ECOLOGY OF PLANT–INSECT INTERACTIONS

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Abstract: Interactions between plants and herbivores are central to all ecosystems. Although numerous types of interaction have evolved, there is considerable overlap in recognition, signal transduction and gene expression events that orchestrate the plant defence. Responses to herbivore damage can be triggered by simple wounding or insect-derived elicitors such as certain enzymes, fatty acid-derived conjugates, other low-molecular-weight aliphatic compounds and peptides generated from degradation of ingested plant material. Early induced responses in host plants are characterised by membrane depolarisation, intracellular [Ca^{2+}] transients and reactive oxygen species production, followed by the activation of protein kinases and a downstream phytohormone networks that coordinate particular responses. Induced and constitutive toxic or harmful secondary metabolites are crucial components of host defence. Also, proteinase inhibitors or threonine deaminase can reduce the nutritional value of the plant tissue by interfering with the herbivore digestion. Synergistic effects between plant constituents ensure efficient mobilisation of defences that cannot be bypassed easily. Emissions of induced volatile compounds influence local and long-range interactions by repelling herbivores and attracting parasites and parasitoids from a distance, thus employing a third trophic level. Moreover, uninfested parts of the same and neighbouring plants can respond to volatiles by upregulating defence-related genes (priming), allowing an accelerated response to actual damage and thereby reducing the metabolic costs that are associated with a fully operational defence system.

Keywords: herbivores; indirect/direct defences; insect-derived signals; signalling; volatiles; wounding

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9.1 Introduction

Plants and insects have coexisted for at least 350 million years. In that period they evolved a variety of different interactions (Gatehouse, 2002). From a plants perspective, these interactions can be beneficial, as in the case of insect-mediated pollination and seed dispersion, or deleterious if they are affected by herbivorous insects. A general scheme of plant biotic interactions including insects is given in Fig. 9.1. A straightforward way to avoid being eaten is to be toxic or at least unpalatable, and chemical defence has been reported for all trophic levels involved in different interactions (Pasteels, 2007). Thus, plants have evolved a large assortment of chemical defences that cause an effective and drastic reduction in insect feeding (Harborne, 1993). The activation of specific defence responses requires efficient recognition of the aggressor, conversion of the perceived signal into downstream signalling cascades and, eventually, the onset of appropriate reactions against the enemy (Maffei *et al.*, 2007). In this chapter, we review our current knowledge of insect-derived compounds that are recognised by the plant and the subsequent signalling processes within the plant. We also describe the diverse indirect and direct strategies that plants have evolved to defend themselves against phytophagous insects. These include physical factors (barriers) as well as the deployment of toxic or harmful phytochemicals that are constitutive or induced. Defensive phytochemicals belong to different classes of secondary metabolites, such as phenylpropanoids, alkaloids and terpenoids, or are fatty acid derivatives. Many of them are airborne compounds that are involved in tritrophic interactions by attracting predators or parasitoids of the feeding herbivore or by mediating inter- and intra-plant communications within or between species. This aspect of plant defence is discussed in terms of options to manipulate and thereby enhance the pest resistance of agricultural important crops.

9.2 Recognition and signalling

Plants are equipped with a sophisticated sensory system to perceive signals from their environment and thereby detect potential enemies and translate such signals into an appropriate biochemical and physiological response. Signal perception in the plant cell may rely, for example, on the presence of specific receptors for chemical signals or on general recognition processes that rely on localised injury of the plant. In principle, herbivory combines two different aspects of the feeding process. First, there is mechanical wounding of the infested tissue and second, the introduction of oral secretions derived from the feeding organism into the wounded tissue. Thus, the attacked plant faces both a mechanical and a chemical challenge (Fig. 9.2).

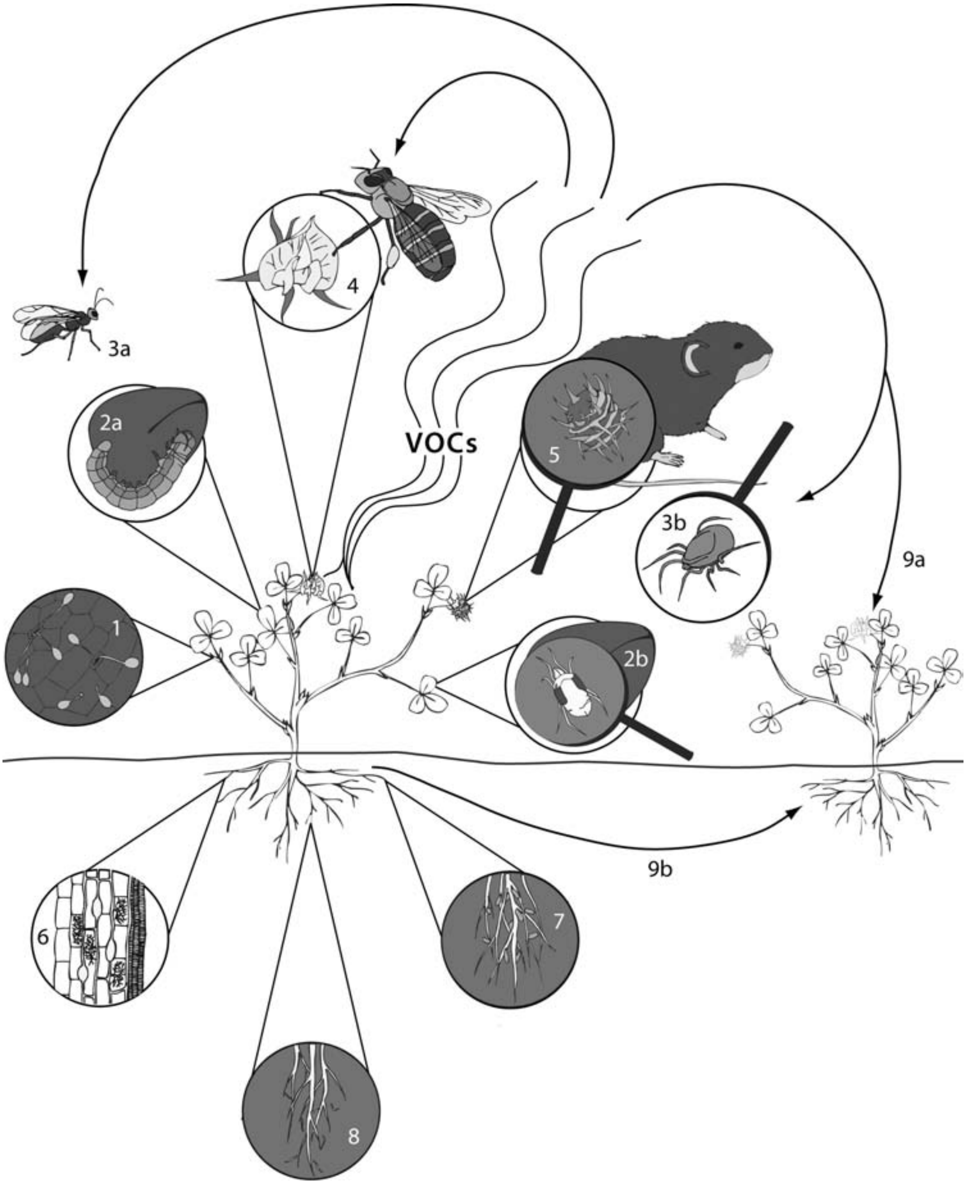


Figure 9.1 Selected biotic interactions of plants: (1) Infection with pathogenic fungi; (2) herbivore attack; (2a) chewing insects, e.g. lepidopteran larvae; (2b) cell content feeders, e.g. spider mites; (3) attraction of parasitoids or predators by herbivore-induced volatiles; (3a) parasitoid wasps attracted to caterpillar-damaged plants; (3b) predatory mites attracted to spider mite-infested plants; (4) attraction of pollinators by floral volatiles and nectar; (5) animals acting as seed dispersers; (6) plant roots colonised by symbiotic arbuscular mycorrhizal fungi; (7) legumes entering into symbiosis with rhizobacteria, resulting in nodulation of the roots; (8) belowground herbivory, e.g. by nematodes. (Courtesy of Margit Leitner.)

Figure 9.2 *Spodoptera littoralis* caterpillar feeding on a barrel medic (*Medicago truncatula*) leaf.

9.2.1 Wounding

Mechanical wounding of plant tissues is an inevitable consequence of insect herbivory, although both the intensity and the extent of damage vary with the mode of feeding, for example sucking (spider mites) or chewing (caterpillars). The impact of injuries on the onset of particular defence reactions has been underestimated for a long time. In almost all studies that investigated the effects of either insect feeding or insect elicitors or both on the emission of organic volatile compounds (see below), control experiments with wounded plants were performed using either a razor (blade) to scratch leaves (Turlings *et al.*, 1990; Schmelz *et al.*, 2001; Spiteller *et al.*, 2001), a forceps to crush leaves (Reymond *et al.*, 2000) or a pattern wheel for puncturing leaf areas (Halitschke *et al.*, 2001). This type of simulated herbivory resulted in induction of genes corresponding to different defence strategies and in upregulation of primary metabolism activities (Cheong *et al.*, 2002; Reymond *et al.*, 2004; Major and Constabel, 2006). However, induction of defence-related volatile emission was not observed. Although it was obvious that wounding had an impact on

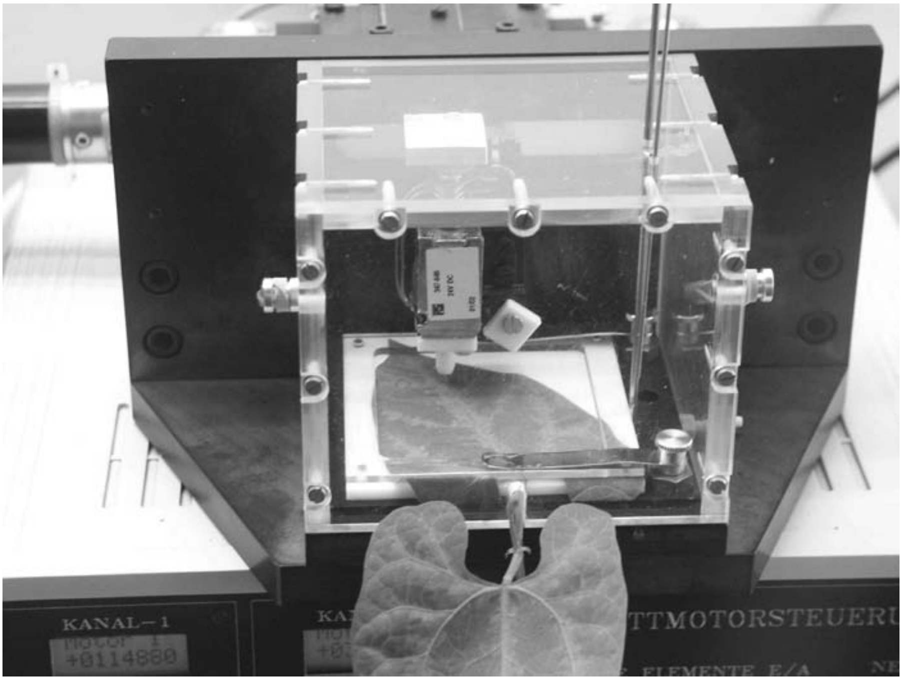


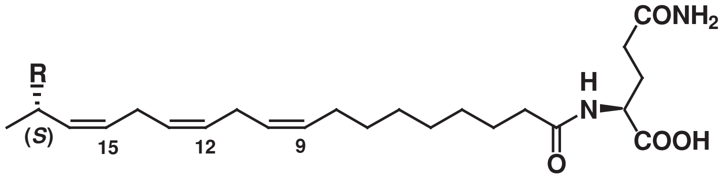
Figure 9.3 MecWorm, the mechanical caterpillar, simulating insect feeding damage of a lima bean (*Phaseolus lunatus*) leaf.

gene expression and physiological responses *in planta*, the question whether or not these treatments were adequate to mimic insect feeding remained unanswered. Herbivorous insects feed on leaves by continuous clipping off pieces of tissue, a process that can be considered as a prolonged series of mechanical woundings (Fig. 9.2). Thus, it is conceivable that plants might recognise and discriminate continuously sustained damage by feeding insects from mechanical wounding that was performed only once. A computer-controlled mechanical larva, MecWorm (Fig. 9.3), has allowed a more faithful simulation of 'herbivory' (Mithöfer *et al.*, 2005). On lima bean (*Phaseolus lunatus*) leaves, nearly the same blend of volatiles could be detected after long-lasting treatment with MecWorm that were known from herbivore damage, although some quantitative differences were observed (Mithöfer *et al.*, 2005). These experiments also revealed that continuous mechanical wounding was sufficient to induce both local and systemic emission of volatiles (Mithöfer *et al.*, 2005). By integrating both parameters of the treatment (the duration and the area damaged), it could be shown that these contribute significantly to the induction of the whole volatile response. However, not all plants investigated have so far responded to MecWorm treatment in the same way as lima bean or cotton (*Gossypium hirsutum*) with the emission of the whole set of volatiles.

It is therefore likely that the relative contributions of mechanical wounding and chemistry of the insects' oral secretions vary in different plant species.

9.2.2 Herbivore-derived signal compounds

As stated above, oral secretions from feeding insects contain herbivore-specific compounds with elicitor-like properties. For instance, enzymes such as glucose oxidase, (Eichenseer *et al.*, 1999), β-glucosidase (Mattiacci *et al.*, 1995) and alkaline phosphatase (Funk, 2001) are elicitor-active compounds. Besides proteins, few low-molecular-weight compounds have been identified as potent elicitors. A representative of a novel class of insect-derived elicitors is volicitin, *N*-(17-hydroxylinolenoyl)-L-glutamine (Fig. 9.4), that was isolated from beet armyworm caterpillars (*Spodoptera exigua*) (Alborn *et al.*, 1997). Meanwhile, other fatty acid–amino acid conjugates have also been described. The basic structures of these signalling compounds could be determined as *N*-acyl-glutamines in which the fatty acid moiety is represented mainly by linolenic acid (C18:3), linoleic acid (18:2) and derivatives thereof (Alborn *et al.*, 1997; Pohnert *et al.*, 1999; Spiteller and Boland, 2003). Some of these compounds exhibited high volatile-inducing activities when added to single wounded plants (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Landolt *et al.*, 1999; Halitschke *et al.*, 2001; Schmelz *et al.*, 2001). In corn (*Zea mays*) plasma membranes, the existence of a high-affinity (K_d~1.3 nM) binding sites for volicitin has been demonstrated (Truitt *et al.*, 2004), suggesting that at least in this species the insect-derived elicitor effects are mediated through a receptor. However, *N*-acyl-glutamines are not generally active as in lima bean and cotton; for example, no induction of volatiles was detected (Spiteller *et al.*, 2001). Interestingly, this corresponds to the high level of purely mechanically induced volatiles in these species. Recently, two more elicitors have been isolated from oral secretions of insects and their structures elucidated. Inceptins, disulfide-bridged peptides representing proteolytic fragments of chloroplastic ATP synthase γ-subunit (Fig. 9.4), were identified in *Spodoptera frugiperda* larval oral secretions (Schmelz *et al.*, 2006). Inceptins triggered ethylene (ET) production, increased the levels of salicylic acid (SA) and jasmonic acid (JA), and mediated plant perception of herbivory through the induction of various defences in cowpea (*Vigna unguiculata*). Clearly, inceptins function as signals initiating specific plant responses to insect attack. Whether insects other than *S. frugiperda* can generate inceptins remains to be elucidated. Caeliferins, compounds found in the grasshopper species *Schistocerca americana*, are composed of saturated and monounsaturated sulphated α-hydroxy fatty acids in which the ω-carbon is functionalised either as a sulphated hydroxyl or as a carboxyl conjugated via an amide bond to glycine (Fig. 9.4; Alborn *et al.*, 2007). Caeliferins induced volatile emission in corn, and it will be interesting to learn whether these compounds are widespread insect-derived elicitors or restricted to certain taxa. An additional type of elicitor was identified in the pea weevil (*Bruchus pisorum*) that comes in contact with the host plant,

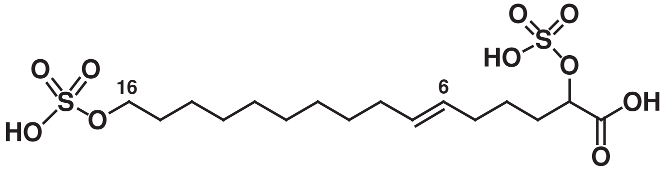


R = H, OH (volicitin)
R = O-PO_3H
R = O-acyl (C_{18}, C_{16})

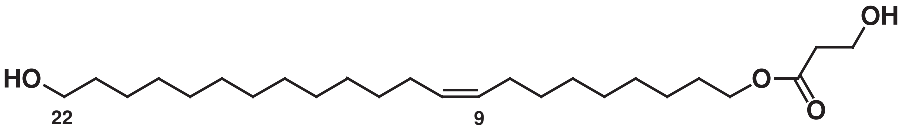
N-**Acyl-glutamines**

N-Ile-Cys-Asp-Ile-Asn-Gly-Val-Cys-Val-Asp-Ala-*C*

***Vu*-Inceptin**



Caeliferin A



Bruchin A

Figure 9.4 Herbivore-derived elicitors. *N*-Acyl-amino acid conjugates: volicitin, *N*-(17-hydroxylinolenoyl)-L-glutamine; inceptins, proteolytic peptides of the chloroplastic ATP synthase γ-subunit, *Vu*: *Vigna unguiculata*; caeliferins: caeliferin A16:1, (*E*)-2,16 disulfooxy-6-hexadecenoic acid; bruchins: bruchin A, (*Z*)-9-docosene-1,22-diol, 1-(3-hydroxypropanoate)ester.

pea (*Pisum sativum*), during oviposition. The elicitors, known as bruchins, are long-chain diols that are mono- and diesterified with 3-hydroxypranoic acid (Fig. 9.4; Doss *et al.*, 2000). Bruchins initiated neoplastic growth on pods in certain genotypes, lifting the eggs out of the oviposition site. This hinders larvae from entering pod tissues, presents them to enemies and makes them vulnerable to desiccation (Doss *et al.*, 2000). For all these insect-derived signalling molecules, we need to elucidate how the different host plants recognise them

to finally understand their particular modes of action. Do they, for example, act directly on the plant membranes or interact with specific binding sites or receptors in the plant that subsequently transduce defence signals.

9.2.3 Signal transduction

Since many recent reviews on plant–insect interactions have already covered the role and hierarchy of early signalling events such as (a) changes of plasma membrane potentials (V_m), (b) intracellular [Ca^{2+}] transients, (c) protein-phosphorylation/-dephosphorylation cascades, as well as the generation of (d) hydrogen peroxide and (e) phytohormones (JA and other oxylipins, SA and ET) (Murphy *et al.*, 1999; Arimura *et al.*, 2005; Lorenzo and Solano, 2005; van Loon *et al.*, 2006; Maffei *et al.*, 2007), we refer the reader to those papers and Fig. 9.5 for details.

Plant defences against herbivory are mediated by a complex network of interacting signal transduction pathways downstream of sensors/receptors that recognise the attacking herbivore. Phytohormones play an important role in the transduction of signals. The three major plant hormones, SA, JA and ET, are important in the defence of both monocots and dicots. Gene expression and other studies have shown that the SA pathway is activated primarily in response to biotrophic pathogens or insects that cause minimal tissue damage such as phloem-feeding aphids (Walling, 2000). In contrast, the JA/ET pathway is induced in response to necrotrophic pathogens, wounding and tissue-damaging insect feeders (Kempema *et al.*, 2007). Changes in gene expression underlying inducible responses to herbivory are complex, and studies of responses to herbivory and mechanical wounding suggest a similar pattern of multiple independent, but networked defence response pathways (Korth and Dixon, 1997; Reymond *et al.*, 2000; Walling, 2000; Hui *et al.*, 2003; Puthoff *et al.*, 2003; Reymond *et al.*, 2004; Voelckel and Baldwin, 2004). However, changes in plant gene expression after herbivory do not always reflect those after jasmonate or wounding treatments (Reymond *et al.*, 2000, 2004). Also, herbivores with different feeding modes induce both unique and overlapping changes (De Vos *et al.*, 2005). JA protects the plant from insect infestation and necrotrophic pathogens that kill the host cell before feeding. This hormone activates the regulatory F-box protein COI1 that is part of the E3 ubiquitin ligase-containing complex SCF^{COI1} which then derepresses JA-responsive genes involved in plant defence (Chini *et al.*, 2007; Thines *et al.*, 2007). The time course for accumulation of individual phytohormones is different and can be assessed after extraction and derivatisation by gas chromatography-mass spectrometry methods (Schmelz *et al.*, 2003b; Schulze *et al.*, 2006). The average resting level of JA was found to be 10–40 ng g^{-1} fresh weight and increased 10–15-fold within the first 1–2 h after damage in various plants (Baldwin *et al.*, 1997; Schmelz *et al.*, 2003b; Schulze *et al.*, 2006). The amounts of its biosynthetic precursor, 12-oxophytodienoic acid,

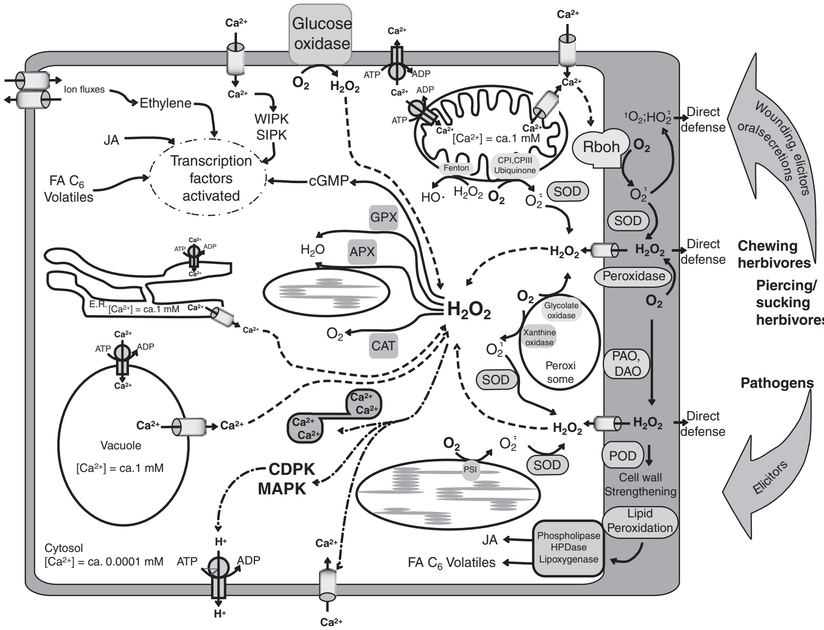


Figure 9.5 Model for herbivore- and pathogen-initiated signalling in plants. Herbivore feeding on plants causes massive damage to leaf tissues and depends on both wounding and release of oral secretions and regurgitant. Recognition of elicitors and/or airborne volatiles prompts rapid responses at the physiological, biochemical and molecular levels. An early event occurs at the plant cell wall and plasma membrane with the production of reactive oxygen species (ROS). The activity of plasma membrane-localised NADPH oxidase (Rboh) enzymes catalyses the production of $O_2^{\bullet-}$ by the one-electron reduction of oxygen using NADPH as electron donor. Dismutation by superoxide dismutase (SOD) in the cell wall yields H_2O_2 that can either enter the cell through peroxiporins or act directly against the invading biotroph. In the apoplast, peroxidases (POD), diamine- (DAO) and polyamine (PAO) oxidases also generate H_2O_2. Lipid peroxidation caused by ROS generates substrates for phospholipase, hydroperoxide lyase and lipoxygenase, with the generation of green leaf volatiles and jasmonic acid (JA). In the protoplast, H_2O_2 can be generated by plant cell organelles and cytosolic enzymes. Production of H_2O_2 depends on the activity of peroxisomal glycolate oxidase, whereas the superoxide anion generated by peroxisomal xanthine oxidase is transformed to H_2O_2 by cytosolic SOD. The latter also reduces superoxide generated by photosystem I in the chloroplasts and complexes I and III and ubiquinone in the mitochondria. Glucose oxidase, either endogenous or introduced in herbivore oral secretions, also generates H_2O_2. In the cytosol, H_2O_2 is reduced to water by the action of glutathione peroxidase (GPX), catalase (CAT) and plastidial ascorbate peroxidase (APX). H_2O_2 induces influx of Ca^{2+} in the cytosol and the efflux of Ca^{2+} from internal stores triggers H_2O_2 production. H_2O_2 production can activate MAPK (mitogen-activated protein kinase) cascades and CDPKs (calcium-dependent protein kinases) that might activate plasma membrane H^+-ATPases and alter the plant cell redox status, thus triggering subsequent signals (such as cGMP), which in turn activate transcription factors. Cytosolic homeostasis of Ca^{2+} is maintained by the activity of primary pumps operating in the tonoplast, plasma membrane and organellar membranes, as well as channel activities. The transient cytosolic increase in $[Ca^{2+}]$ leads to an activation of wound-induced protein kinases (WIPKs), which in turn could deregulate transcription factors. Activation of transcription factors can also be triggered by JA and fatty acid-derived C_6 volatiles as well as by ion flux-induced ethylene production. There are several parallels between herbivore-induced and pathogen-induced plant cell responses as described (Maffei *et al.*, 2007). SIPK, salicylate-activated protein kinase

also increased (two- to fivefold) as did those of ET, while levels of abscisic acid and indole acetic acid are often reduced (Schmelz *et al.*, 2003b).

JA and SA function as signalling molecules that mediate induced plant responses towards herbivory and pathogen infections resulting in the activation of distinct sets of defence genes (Reymond and Farmer, 1998; Turner *et al.*, 2002). Besides free JA, also conjugates with the amino acid isoleucine, and probably other amino acids, are involved in signalling (Krumm *et al.*, 1995; Staswick and Tiryaki, 2004; Kang *et al.*, 2006; Thines *et al.*, 2007; Walter *et al.*, 2007). Inhibiting the performance of JA or SA renders plants more susceptible to herbivore damage (McConn *et al.*, 1997; Halitschke and Baldwin, 2003; Paschold *et al.*, 2007). The interaction of various pathways in the networks is assumed to result in an integrated overall response that induces a characteristic defence pattern. Several oxylipins likely act as master switches for herbivore-stimulated plant responses. JA and SA often act antagonistically and both are required for the induced response following herbivore feeding or pathogen attack (Ozawa *et al.*, 2000a; Engelberth *et al.*, 2001; Cipollini *et al.*, 2004). In addition, antagonistic or synergistic cross-reactions with other regulators, such as ET, seem to control and coordinate a characteristic activation of defence-related compounds and genes leading to defence (Kahl *et al.*, 2000; Winz and Baldwin, 2001). Recently, it was shown in *Medicago truncatula* that ET contributes to herbivore-induced terpenoid biosynthesis in at least two ways: by modulating early signalling events such as cytoplasmic Ca^{2+} influx and the downstream JA-dependent biosynthesis of terpenoids. However, most observations are still descriptive.

Upstream of JA it is likely that protein kinases mediate herbivore-induced signalling. A subclass of MAPK (mitogen-activated protein kinase), the wound-induced protein kinase (WIPK), was essential for JA formation and JA-induced responses, since *WIPK*-silenced plants were unable to produce JA or methyl-JA after wounding. The loss of WIPK function resulted in increased accumulation of SA and its β-glucoside after wounding. Moreover, WIPK was reported to elicit transcription of a gene for a ω-3 fatty acid desaturase (FAD7) which catalyses the conversion of linoleic acid to linolenic acid, a precursor of JA (Kodama *et al.*, 2000). Thus, WIPK may be an early activator of the octadecanoid pathway.

Recently, it was demonstrated that *Manduca sexta* oral secretions activated the MAPKs WIPK and SIPK (salicylate-activated protein kinase) in leaves of *Nicotiana attenuata* (Wu *et al.*, 2007). Transcriptional analyses of *N. attenuata* suggested that *SIPK* and *WIPK* mediate the *M. sexta* oral secretion-elicited accumulation of many defence-related genes including three *MAPK*s, four calcium-dependent protein kinases (*CDPKs*) and transcription factors of the *WRKY* type (Wu *et al.*, 2007). After oral secretion elicitation, a fast mobile signal moved to certain undamaged parts of the elicited leaf and activated MAPK signalling and downstream responses (Wu *et al.*, 2007). However, this signal was not able to induce a systemic response beyond the affected leaf. These data suggest that the attack by a herbivore results in an acute localised

activation of SIPK, WIPK and the MAPK cascade, resulting in the production of JA and ET, which might then prime other leaves to respond to larval attack. It will be interesting to explore where the regulated kinases are positioned within the different signal transduction pathways in plant cells and, more specifically, whether they represent a component-relaying herbivore-mediated changes in ion fluxes to specific transcription factors involved in the activation of defence-related genes. Besides the still elusive chemical signals mediating systemic induction of defences, it is conceivable that electrical phenomena such as fast action potentials or slow waves potentials (also called variation potentials) (Davies, 2004) provide the link to distant physiological responses.

9.3 Defence strategies

As primary producers, plants are eaten by a wide range of herbivores, including nematodes, arthropods, molluscs and mammals (Fig. 9.1), and have evolved a wide spectrum of physicochemical strategies to defend themselves against attacking organisms in their environment. Plant defence mechanisms can be classified as constitutive and induced. The former include morphological and structural features as well as constitutively produced defense compounds. Induced defences are by contrast activated after herbivore or pathogen attack and can be classified into direct and indirect modes of self-protection. Direct defences directly target the performance or survival of the attacking organism and include the induced accumulation of toxins or feeding deterrents, fortification of cell walls and the hypersensitive reaction. Indirect defences are mostly associated with defence against herbivores and protect the plant through manipulation of the behaviour of predators or parasitoids of an attacking herbivore. Thus, indirect defences employ a third trophic level by attracting natural enemies of the plant's attacker (Fig. 9.1) (Takabayashi and Dicke, 1996; Kessler and Baldwin, 2001). Mechanisms of indirect defence also include the offer of accommodation to carnivores, as, for example, by so-called domatia, and the offer of alternative food sources, such as extrafloral and floral nectar or food bodies (Heil and McKey, 2003; Kost and Heil, 2005). Both direct and indirect defences can be constitutive or induced.

9.3.1 Constitutive defences

Constitutive physical barriers such as thorns or prickles can deter larger herbivores, whereas thick cell walls or cuticles, high levels of lignification or the accumulation of resin can hinder penetration by bacteria, fungi and smaller herbivores. Trichomes present a mechanical barrier. They can also be secretory structures, producing and storing antimicrobial compounds, feeding or egg deposition deterrents or toxins. Plant can also resist herbivore attack

by producing digestibility reducers including tannins, resins, proteinase inhibitors (PIs) and silica. These compounds can delay herbivore development by reducing its digestive efficiency, thus lowering resistance to disease and limiting fecundity (Price *et al.*, 1980).

The way in which plants store toxins is often crucial to their effectiveness. Certain plant species accumulate toxins in resin ducts or glandular trichomes (Duke *et al.*, 2000), and such toxins can be released in large amounts once these structures are ruptured by herbivore feeding or movements on the plant surface. Since many constitutive defence compounds are toxic to the plant itself, the plant must generate and store such substances without poisoning itself. One commonly adopted strategy is to store toxins as inactive precursors, for example as glycosides (Jones and Vogt, 2001), and to separate them from activating hydrolases. In this respect, one of the best known examples is the glucosinolates (Rask *et al.*, 2000; Wittstock and Halkier, 2002). These compounds, present in the taxon Capparales, are compartmentalised and thus separated from their activating enzyme, a thioglucosidase myrosinase. Whereas glucosinolates are found in many plant tissues, myrosinase is localised only in scattered cells. Upon tissue damage, glucosinolates and myrosinase come in contact to form an unstable aglycone that rearranges into a variety of active compounds, typically isothiocyanates and nitriles. The biological activity of isothiocyanates released upon glucosinolate hydrolysis ('the mustard oil bomb') became apparent in a study showing that larvae of the lepidopteran generalist *Trichoplysia ni* avoided *Arabidopsis thaliana* ecotypes that produced isothiocyanates upon glucosinolate hydrolysis and fed instead on ecotypes that produced nitriles from glucosinolates (Lambrix *et al.*, 2001).

Mechanisms of toxicity in different plants depend on the particular defensive compounds. In general, it includes membrane disruption, inhibition of nutrient and ion transport or signal transduction, inhibition of metabolism or the disruption of hormonal control of physiological processes (Gatehouse, 2002; Wittstock and Gershenzon, 2002; Mumm and Hilker, 2006). For example, saponins disrupt cellular membranes (Osbourn, 1996), hydrogen cyanide that is released from cyanogenic glycosides inhibits cellular respiration (Wittstock and Gershenzon, 2002), cardenolides are specific inhibitors of the Na^+/K^+-ATPase (Wittstock and Gershenzon, 2002) and the polyacetylene, cicutoxin, was shown to act by prolonging the repolarisation phase of neuronal action potentials, presumably by blocking voltage-dependent potassium channels (Wittstock *et al.*, 1997). The modes of action of many other toxins need to be elucidated.

Although constitutive defences often enable specific plant hosts to be resistant to specific insect pests (Gatehouse, 2002), in the arms race between insects and plants herbivorous insects have developed counter-adaptations that allow them to feed on chemically defended plants without negative effects. One example is the performance of larvae of the lepidopteran specialist insect *Pieris rapae* that are adapted to the glucosinolate/myrosinase defence

system of Brassicaceae hosts. In this case, an insect gut protein manipulates the hydrolysis reaction towards the formation of nitriles instead of isothiocyanates, the nitriles then being excreted with the feces (Wittstock *et al.*, 2004).

An example for indirect constitutive defence is given by Central American *Acacia* species that secrete extrafloral nectar to attract and nourish ants which in turn defend the host plant against herbivores (Heil and McKey, 2003). This form of indirect defence can be inducible as well as constitutive. In the latter case the plants are obligatory, inhabited by symbiotic ants. Phylogenetic analysis revealed that the inducibility of extrafloral nectar secretion is the ancestral (plesiomorphic) state, and the constitutive nectar flow represents the derived (apomorphic) state within the genus *Acacia* (Heil *et al.*, 2004).

9.3.2 Induced defences

In induced processes, rather than in the case of constitutive defences, recognition of an attacking insect and the subsequent alarm signalling is a prerequisite for rapid and efficient defence, and many of the plant strategies mentioned above can be constitutive, inducible or both. Strikingly, many forms of induced defence are not restricted to local responses at the wounding site, but can be detected systemically throughout the plant. Thus, induced defences also involve the synthesis and accumulation of various secondary metabolites that influence insect attraction/deterrence and inhibit insect growth and development. In addition, proteins such as proteinase inhibitors (PIs), polyphenol oxidases (PPOs) and threonine deaminase (TD) that inhibit insect digestive enzymes and/or decrease the nutritive value of the plant tissues are employed in induced direct defences (Baldwin *et al.*, 2001). For example, herbivore attack on *N. attenuata* rapidly increases the production and accumulation of trypsin proteinase inhibitors (TPIs) (Zavala *et al.*, 2004). *Spodoptera exigua* performed better on TPI- or nicotine-deficient plants compared to wild type (Steppuhn and Baldwin, 2007). Interestingly, due to a compensatory feeding response to TPI when nicotine is absent, larvae performed better on nicotine-deficient plants than they did on plants silenced in both defences. The antifeedant toxin nicotine was found to prevent this compensatory response. Thus, *N. attenuata* counters an insect adaptation with a defensive synergism (Steppuhn and Baldwin, 2007). In tomato (*Lycopersicon esculentum*), PIs were tested for their trypsin- and *Helicoverpa armigera* gut proteinase-inhibitory activity in different organs of the plant. Observation in the field revealed that *H. armigera* larvae infested leaves and fruits but not flowers, a fact that has been correlated with the higher levels of PIs in flower tissues (Damle *et al.*, 2005). In barley leaves, wounding experiments led to minimal accumulation of PI activity (16% over controls) compared to that found in plants infested by aphids, where chymotrypsin inhibitor activity showed a twofold increment. By contrast, JA increased only trypsin inhibitory activity both locally and systemically when applied exogenously. These data suggest that two

different mechanisms might regulate the induction of these two types, trypsin inhibitors and chymotrypsin inhibitors (Casaretto *et al.*, 2004). TD serves a dual role in isoleucine biosynthesis *in planta* and threonine degradation in the insect midgut. Purified pTD2 exhibited biochemical properties that are consistent with a postingestive role in defence. In particular, expression of *TD2* in tomato leaves is activated by the JA-signalling pathway in response to herbivore attack, and the ingestion of tomato foliage by specialist (*M. sexta*) and generalist (*T. ni*) insect herbivores triggers proteolytic removal of TD2's C-terminal regulatory domain. This results in an enzyme that degrades threonine without being inhibited through feedback by isoleucine in the gut of the feeding insect (Chen *et al.*, 2007). Thus, protease inhibitors have been proposed as potential defence molecules to enhance insect resistance in crop plants (Abdeen *et al.*, 2005; Fan and Wu, 2005).

Phenolic compounds such as the polyphenol tannins are often correlated with resistance to herbivores, and in some trees their accumulation is induced by previous herbivory. PPO is an enzyme catalysing the oxidation of *o*-diphenolic compounds to *o*-quinones as well as the hydroxylation of monophenols to *o*-diphenols. Wounding of hybrid poplar leaves caused strong induction of PPO activity (Constabel and Ryan, 1998). PPOs are known to reduce the nutritive value of plant tissue for insects and are involved in secondary metabolite production. In sugar beet (*Beta vulgaris*) roots responding to sugar beet root maggot (*Tetanops myopaeformis*) feeding, multiple *PPO* genes were identified due to their induction (Puthoff and Smigocki, 2007).

In response to herbivores, many plants release volatile organic compounds. Volatiles can carry various types of information: (a) for herbivores to localise their host, (b) for indirect defence employing a third trophic level by attracting natural enemies of the plant's attacker and (c) for distant parts of the same plant or neighbouring plants to adjust their defensive phenotype accordingly (Heil and Silva Bueno, 2007). Chemically, volatiles belong to the large group of terpenoids (mono-, di-, sesqui- and homoterpenoids), fatty acid-derived C_6 volatiles and derivatives (e.g. (3*Z*)-hex-3-enal, (3*Z*)-hex-3-enyl acetate), aromatic compounds such as methyl salicylate and indole as well as certain alkanes, alkenes and alcohols (Fig. 9.6; Holopainen, 2004; Arimura *et al.*, 2005). Since the complex blends are fed by different biosynthetic pathways linked to a web of interacting signals, volatiles might be considered as a 'volatilome' that is particularly sensitive to different external triggers. The insect-feeding-induced emission of volatiles has been demonstrated for several plant species (van Poecke and Dicke, 2004), among others corn, *Zea mays* (Turlings *et al.*, 1990); cotton, *Gossypium hirsutum* (Röse *et al.*, 1996); lima bean, *Phaseolus lunatus* (Dicke *et al.*, 1990); *Lotus japonicus* (Ozawa *et al.*, 2000b); tobacco, *Nicotiana attenuata* (Kessler and Baldwin, 2001); and barrel medic, *M. truncatula* (Leitner *et al.*, 2005). Herbivore-induced plant volatiles represent phenotypically plastic responses of plants to herbivory, which result in changes in interactions between individuals in the insect–plant community (Snoeren *et al.*, 2007). Recently, a novel role of herbivore-induced volatiles

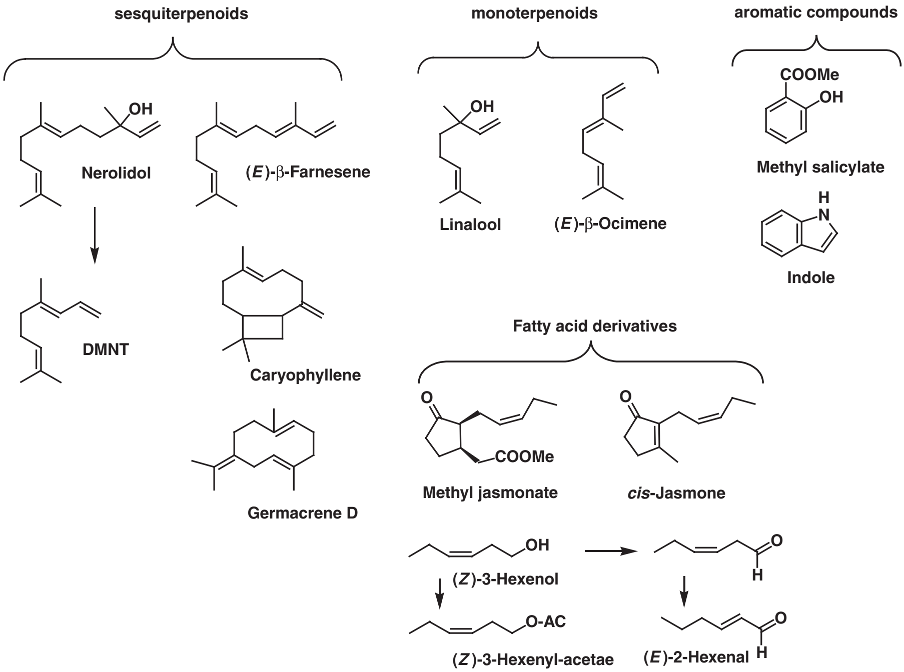


Figure 9.6 Typical herbivore-induced volatiles. DMNT, 4,8-dimethyl-1,3,7-nonatriene.

in parasitoidic foraging behaviour, different from the widely studied attraction at a distance, was introduced by Tentelier and Fauvergue (2007). After visiting heavily aphid (*Aphis gossypii*) infested cucumber (*Cucumis sativus*) plants, parasitoids (*Lysiphlebus testaceipes*) reduced their residence time and attacked fewer hosts in the next patch. This occurred regardless of whether the aphids were actually present on the first plant, indicating that the cue came from the plant. Moreover, the level of infestation of a plant at some distance from the first plant visited affected parasitoid patch exploitation on the second plant in a similar manner, suggesting that the cue was a volatile. In other words, parasitoids adjust their patch exploitation behaviour on a plant according to the herbivore-induced volatiles emitted by the plants they have visited previously or by other plants in the vicinity of those plants. For the plants this parasitoidic behaviour is a source of additional selection pressure, favouring the emission of large amounts of herbivore-induced volatiles: a plant emitting more synomones than its counterparts is likely to attract more parasitoids and to retain them for relatively longer, thereby increasing the number of phytophagous insects attacked on that plant (Tentelier and Fauvergue, 2007).

Induced plant responses often provide reliable information about the identity of the herbivores concerned. At least in some systems, the amounts of

volatile compounds emitted by the plant are correlated with the density of phytophagous insects feeding on that plant (Schmelz *et al.*, 2003a). These volatiles affect the flying behaviour of parasitoids that are attracted towards the most profitable plants, those bearing the largest number of hosts. Animals usually require information about the current state of their habitat to optimise their behaviour. For this, they can use a learning process through which they are continually updated according to the cues they perceive.

Using plants upon which herbivores are feeding and investigating by gas chromatography-electroantennography or gas chromatography-single cell recording the volatile compounds released, it is possible to identify a range of compounds that are electrophysiologically active and which may subsequently prove to be active in behavioural assays as repellents of insect pests. These compounds can also be active in increasing foraging by predators and parasitoids that attack the pests. The compounds involved come from a wide range of biosynthetic pathways, but prominent in these are the isoprenoid and lipoxygenase pathways. For example, monoterpenes, such as (*E*)-ocimene, and sesquiterpenes, such as (−)-germacrene D, can be produced by plants and cause repellence to herbivores.

The induction of volatile emissions not only occurs in response to herbivore feeding on leaves, but can also result from the deposition of insect eggs on plant parts. The known indirect plant defence mechanisms induced by egg deposition act by helping egg parasitoids to locate their hosts. A study of wild tobacco (*N. attenuata*) plants in their natural setting showed that spiking the plants with synthetic versions of several typical volatiles increased consumption of lepidopteran eggs that had been placed on these plants by carnivorous insects and consequently reduced damage by herbivores considerably (Kessler and Baldwin, 2001). For three tritrophic systems (host plants: elm, pine and bean), it was shown that insect egg deposition induced a plant volatile pattern that attracted egg parasitoids, whereas in a further system consisting of *Brassica* (plant host), *Pieris* (herbivore) and *Trichogramma* (carnivore) egg deposition very likely induced the change of plant surface chemicals, thus arresting the egg parasitoids by contact cues in the vicinity of the eggs (Hilker and Meiners, 2006, and references cited therein).

Even below ground the emission of volatiles is an efficient trait. In maize roots, the sesquiterpene (*E*)-β-caryophyllene is necessary to attract entomopathogenic nematodes to roots damaged by the ferocious maize pest *Diabrotica virgifera virgifera*. Maize varieties that lack this signal are far more vulnerable to the pest (Rasmann *et al.*, 2005). In general, volatiles can be considered as infochemicals that mediate many interactions in a plant–insect community both above and below ground (Bezemer and van Dam, 2005). Belowground interactions and their putative impact on aboveground events and activities (and vice versa) are a topic of increasing interest and worth more intensive investigation (van Dam *et al.*, 2003; Bruce and Pickett, 2007). Also, knowledge of root responses at the molecular level will be useful in the development of alternative root insect control strategies for crop plants.

In response to herbivory, damage or increased JA levels, lima beans not only release volatiles but also secrete extrafloral nectar, nectar that attracts predatory arthropods, mainly ants, and therefore serves as an indirect defence (Heil, 2004). Wild lima beans significantly benefited from induced increased nectar production in terms of less leaf damage and higher growth rates and seed production, respectively (Kost and Heil, 2006). Moreover, volatiles released by damaged lima bean leaves could induce extrafloral nectar in neighbouring plants (Kost and Heil, 2006) as well as in undamaged leaves of the same shoots (Heil and Silva Bueno, 2007).

9.4 The cost of defence to plants

A fundamental dilemma that plants face is whether to allocate limited resources to grow fast enough to compete with other plants or to invest these resources to maintain defence. For instance, the deployment of secondary metabolites, in particular terpenoids, as antiherbivore defences is thought to be costly for plants in terms of primary metabolism (Gershenzon, 1994). Defence costs provide a plausible explanation for why plants in nature have not evolved even better defences against pathogens and herbivores; however, evidence for defence costs is often lacking (Siemens *et al.*, 2003). In general, investment in defence, although making the plant ecologically successful, is thought to reduce plant fitness in an enemy-free environment. Such fitness costs may result (a) from allocation costs, i.e. allocation of limited resources to defence which then cannot be used for growth or other physiological processes (Herms and Mattson, 1992); (b) from constitutive costs of inducible defences that might occur if plants have attacker-detection pathways, defence precursors and storage vesicles prepared, all of which require constitutive allocation of energy and resources (Purrington, 2000); (c) if employed defence traits are toxic to the plant, their constitutive expression might impose a significant metabolic tax (Baldwin and Callahan, 1993); (d) if defence traits that are directed against a certain pest might affect beneficial interactions of the host plant with other organisms like insects pollinators or symbiotic microorganisms (Heil, 2001); (e) incompatibility with defences directed against other groups of enemies during simultaneous attacks (Bostock *et al.*, 2001). From an evolutionary perspective, any organism should respond to those trade-offs in a way that maximises its reproductive output and minimises any investment in non-reproductive traits that are, however, essential for its survival. One example for such an evolutionarily optimised response that is considered as a cost-saving strategy is induced plant defences which are activated only in case of an herbivore attack (Berenbaum, 1999; Baldwin and Hamilton, 2000; Dicke and Hilker, 2003). Induced defences such as the emission of volatiles and the secretion of extrafloral nectar by plants are two widespread indirect defence traits involved in mediating the interaction between herbivore-damaged plants and the third trophic level (Paré and Tumlinson, 1999; Heil

et al., 2001). Both defences are neither consistent throughout their lifetime nor evenly distributed within a plant (Zangerl and Rutledge, 1996).

However, 'just-in-time' defence strategies have a disadvantage because plants are unprotected until the induced defence is established. In the worst case this can take days. Clearly, the selective advantage of induced over constitutively expressed defence traits demands some explanation. If the onset of defences incurs significant fitness costs, the drawbacks of the delay might be overcome by the fitness benefit of not investing such costs when defence is not needed (Heil and Baldwin, 2002). This cost–benefit explanation has been widely applied to induced resistance against herbivores (Baldwin and Preston, 1999).

In contrast to our understanding of defence mechanisms, only little is known about traits that allow plants to tolerate herbivore damage. All defences can limit the extent of damage but even well-defended plants lose large amounts of tissue when attacked by adapted herbivores. In such a situation, plants would benefit from a tolerance strategy that minimises the fitness consequences of tissue loss to herbivores (Tiffin, 2000; Strauss *et al.*, 2003), or from combining both strategies of defence and tolerance (Tiffin and Rausher, 1999; Mauricio, 2000). A convincing example was shown recently for *N. attenuata* that was infested by the nicotine-adapted *M. sexta* larvae. Upon attack, *N. attenuata* initiated its repertoire of induced defences, but also started to store newly fixed carbon in the roots. By using ^{11}C-photosynthate labelling in combination with carbohydrates and enzyme measurements, rapid changes in sink–source relations were demonstrated after simulation of *M. sexta* attacks, which increased the allocation of sugars to roots. This process was regulated by GAL83, the β-subunit of an SnRK1 (SNF1-related kinase) protein kinase (Schwachtje *et al.*, 2006). Since root tissue is safe from the leaf-feeding herbivore, carbon storage there may be a means of shielding from loss. Once allocated to the roots, the carbon can be reused when needed or after infestation ceases.

9.5 Airborne communication

Plants are expert in the production and release of airborne signals and many plant–insect interactions rely on airborne communication. The controlled emission of volatiles blends from leaves during herbivore attack can help plants to deter herbivores or attract their predators from higher trophic levels. It can also provide information about the status of attack of the infested plant that can be used (eavesdropping) by neighbouring plants of conspecific or another species (Baldwin and Schultz, 1983; Arimura *et al.*, 2000; Farmer, 2001; Karban, 2001). This of course suggests that plants are able not only to emit volatiles but also to perceive or recognise them. Unfortunately, the mode and mechanisms underlying volatile recognition are completely unknown. In contrast, evidence is accumulating that volatiles are strongly implicated

in plant–plant communication processes. After wounding and/or herbivore damage, some plants emit methyl-JA, which is known to effectively turn on defences. For example, placing intact tomato plants and sagebrush branches together in a closed chamber resulted in the accumulation of defence-related PIs in the leaves of tomato. This was found to be due to the release of methyl-JA from the sagebrush (Farmer and Ryan, 1990). Also, *N. attenuata* plants appeared to suffer less herbivory in the vicinity of wounded sagebrush (Karban *et al.*, 2000). However, not all plants release methyl-JA, calling into question its role as a general volatile defence signal. When infested by herbivorous mites, cotton seedlings produced volatile cues that elicited attraction of predatory mites. Experiments were carried out showing that downwind uninfested conspecific seedlings are affected by these volatiles. It was found that the rate of oviposition by herbivorous mites (*Tetranychus urticae*) was reduced on seedlings exposed to volatiles from infested seedlings. In addition, predatory mites (*Phytoseiulus persimilis*) were attracted by exposed uninfested seedlings. These results indicated that uninfested plants are somehow protected against herbivore attack when exposed to airborne chemicals released by their infested neighbours (Bruin *et al.*, 1992). Many plants respond to volatiles by changing transcription patterns of defence-related genes (Bate and Rothstein, 1998; Arimura *et al.*, 2000; Farag *et al.*, 2005; Paschold *et al.*, 2006), and they may increase the production of defence-related plant hormones such as JA and other volatiles (Engelberth *et al.*, 2004; Ruther and Kleier, 2005) of PIs (Tscharntke *et al.*, 2001), phenolic compounds (Baldwin and Schultz, 1983) or extrafloral nectar (Choh *et al.*, 2006; Kost and Heil, 2006; Frost *et al.*, 2007). The results of these studies demonstrate that volatiles can serve as an external signal for both within-plant and plant–plant communication and even between different species. Nevertheless, there are limitations for airborne plant–plant communication, in particular the distance between emitter and receiver plant. Recent field studies demonstrated that pairs of sagebrush plants that were at maximum 60 cm apart, as given for most individuals in the field, were still influenced by experimental clipping of a neighbour. However, air contact was essential for communication and any treatments that reduced airflow between neighbouring individuals, due to either wind direction or bagging, prevented induced resistance (Karban *et al.*, 2006). In contrast, communication between sagebrush and *N. attenuata* was successful only up to a distance of 15 cm (Karban *et al.*, 2003). Not surprisingly, airflow was also necessary for systemic induced resistance among branches within an individual (Karban *et al.*, 2006). Interestingly, not only infested or damaged plants are able to interact via volatiles. Aerial allelopathy between different cultivars of barley (*Hordeum vulgare*) plants has been shown to alter the biomass allocation in neighbouring plants. While the total dry weight was not significantly affected, there was an increase of the specific leaf area (Ninkovic, 2003). Moreover, barley plants exposed to volatiles from neighbouring plants were less readily accepted by aphids (*Rhopalosphum padi*) when specific cultivars of barley were combined. This effect could be

confirmed under field conditions (Ninkovic *et al.*, 2002). Exposure to volatiles from creeping thistle, *Cirsium arvense*, caused barley plants to become more attractive to ladybird (*Coccinella septempunctata*) (Ninkovic and Pettersson, 2003). Significant changes in leaf temperature of exposed plants to allelobiotic volatiles showed that the allelopathic effect is systemic (Pettersson *et al.*, 1999). If such induced allelopathy mechanisms follow the same pattern as the insect- or microbe-induced defences, this may offer new perspectives in plant protection by using mixed cultivars or by mixing appropriate crop species.

9.6 Priming and manipulations to enhance pest resistance

Plants can acquire enhanced resistance to pathogens after infection with necrotising pathogens or insect pests, non-pathogenic root-colonising bacteria as well as by treatment with SA and many other natural or synthetic compounds. Such induced resistance is associated with a faster and stronger activation of defences upon pathogen or insect attack (Conrath *et al.*, 2002, 2006), and the physiological state is referred to as priming (see Chapter 11). Engelberth *et al.* (2004) reported that corn plants were primed by green leafy volatiles [e.g. (Z)-3-hexenal, (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate] released from damaged plants. Exposure to such volatiles caused undamaged corn plants to produce JA and terpenes more intensively and rapidly, respectively, in response to caterpillar damage compared to plants that were damaged without pretreatment. However, no such priming effects were observed in tobacco plants (Paschold *et al.*, 2006), which also did not respond to volatile methyl-JA at concentrations similar to those occurring under natural conditions (Preston *et al.*, 2004). Recently, it was shown in lima beans that pretreatment with volatiles originating from infested neighbouring plants enhanced the extrafloral nectar production after spider mite (*T. urticae*) infestation (Choh and Takabayashi, 2006). Also, in field experiments in Mexico, lima bean plants that had been exposed to a synthetic volatile blend responded to subsequent leaf damage with increased extrafloral nectar secretion (Kost and Heil, 2006). Clearly, volatiles primed the plants to respond more efficiently once they were attacked. This phenomenon is seen not only in plant–plant communication but also in within-plant signalling by volatile compounds. Again, field studies with lima beans demonstrated that volatiles primed extrafloral nectar secretion in undamaged leaves of the same shoot (Heil and Silva Bueno, 2007). Undamaged leaves of hybrid poplar (*Populus deltoides* × *nigra*) exposed to volatiles released from herbivore-wounded leaves on the same stem with little or no vascular connection were also primed and hence had elevated defensive responses to feeding by gypsy moth larvae (*Lymantria dispar*) compared to leaves that did not receive volatiles (Frost *et al.*, 2007). Priming events have also been documented at the molecular level. In maize, a differential hybridisation screen for inducible genes upon attack by *S. littoralis* caterpillars identified ten defence-related genes that are

responsive to wounding, JA or caterpillar oral secretion (Ton *et al.*, 2007). Exposure to volatiles from caterpillar-infested maize plants could not activate these genes directly. However, a subset of them was primed for earlier and/or stronger induction upon subsequent defence elicitation (Ton *et al.*, 2007). This study suggests that volatile-induced priming might target a subset of JA-inducible genes and links these responses to enhanced levels of direct and indirect resistance against insect attack. In communication between different plant species, it was shown that priming, rather than direct elicitation, of native *N. attenuata* induced chemical defences by a sagebrush (*Artemesia tridentata* ssp. *tridentate)*-released volatile blend. Accelerated production of TPIs was observed when *M. sexta* caterpillars fed on plants previously exposed to clipped sagebrush (Kessler *et al.*, 2006).

Priming for defence against pathogens, and very likely against herbivores, has been shown to produce considerably fewer costs than induction of direct defence, and the benefits derived from this priming outweigh the costs under conditions of disease pressure (van Hulten *et al.*, 2006). Plants that are merely primed for enhanced defence after the perception of distress signals, for example via volatiles from nearby plants or adjacent leaves, are better protected in an environment of herbivore pressure, without suffering the costly energy investment in defence maintenance.

The phenomenon of volatile-induced priming against insects also fits in the ecological context of costs and benefits. Additional agronomical benefits would be expected if emissions of appropriate volatiles were to be engineered in crop plants (Turlings and Ton, 2006). Bioengineering of vegetative plant volatiles that have an impact on insect behaviour might represent a viable strategy to enhance pest resistance of plants. In principle, metabolic engineering of volatiles can be achieved through the modification of existing pathways, for instance by up- or downregulation of one or more biosynthetic steps or by redirection of metabolite fluxes to a desired compound by blockage of competing pathways. Another possibility would be the introduction of new gene(s) or branch pathways that are normally not active in the host plant. The first successful alteration of volatile-based direct plant defence was developed by overexpressing a dual linalool/nerolidol synthase (FaNES1) from strawberry (*Fragaria* × *ananassa)* in chloroplasts of *A. thaliana*. In dual choice assays, it was demonstrated that linalool and its derivatives produced by the transgenic plants significantly repelled the aphid *Mysus persicae*, an agricultural pest (Aharoni *et al.*, 2003). Direction of FaNES1 to another compartment, the mitochondria that contain the sesquiterpene precursor FPP, led to the formation of (3*S*)-(*E*)-nerolidol and its derivative, the C_{11} homoterpene DMNT. Both volatiles attracted the carnivorous predatory mites *P. persimilis* and thus improved plant indirect defence (Kappers *et al.*, 2005). Indirect defence of *A. thaliana* was also improved by overexpression of terpene synthase TPS10 from maize which is responsible for the formation of (*E*)-β-farnesene, (*E*)-α-bergamotene and a variety of other herbivore-induced sesquiterpenes (Schnee *et al.*, 2006). A similar approach using transgenic plants but a

different strategy was used by Beale *et al.* (2006), who overexpressed a gene for (*E*)-β-farnesene synthase from *Mentha* × *piperita* in *Arabidopsis* plants. The higher level of (*E*)-β-farnesene had two positive effects. First, it enhanced attractiveness of the plant to the parasitic wasp *Diaeretiella rapae*, and second, it elicited repellent and alarm responses that caused aphids (*M. persicae*) to stop feeding and move away from the host (Beale *et al.*, 2006). However, (*E*)-β-farnesene needs to be very pure without high level of other terpenoids in the background to be effective.

Transgenic *Arabidopsis* plants with altered biosynthesis of green leaf volatiles showed striking responses when subjected to herbivory. In hydroperoxide lyase (HPL) overexpressing plants, a significant increase in green leaf volatiles production was observed after herbivory by *Pieris rapae* larvae compared with *Arabidopsis* wild-type controls. Moreover, these transgenic plants were more attractive to the wasp *Cotesia glomerata*, leading to higher mortality of the herbivores. By contrast, in *HPL* antisense *Arabidopsis* plants, green leaf volatiles formation decreased and attracted fewer parasitoids than the wild-type control plants (Shiojiri *et al.*, 2006).

These data indicate that genetic modification of green leaf volatiles as well as terpenoid biosynthesis could be a useful approach to improve plant and, in particular, crop resistance against herbivory.

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Chapter 10

LIPID SIGNALS IN PLANT–PATHOGEN INTERACTIONS

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Abstract: Lipids influence multiple stages of plant–pathogen interactions including communication between the host and the microbe, activation and implementation of plant defenses, and the pathogen life cycle. Some pathogens recognize plant lipid-derived signals to identify an appropriate host. Other pathogens depend on the host for lipids as essential molecules or as developmental signals. In contrast, plants have evolved mechanisms to recognize microbial lipids and this can lead to elicitation of defense responses. In several cases, lipid modifications target plant signaling proteins and microbial elicitors to plant cell membranes where defense signaling is initiated. The membrane also provides a reservoir from which biologically active signaling lipids, or their precursors, are released by a variety of hydrolytic enzymes. A large number of lipid-modifying enzymes are involved in the synthesis of signaling lipids. This chapter focuses on progress made in recent years on lipids, lipid signaling, lipid-modifying enzymes and lipid-transfer proteins that influence the outcome of plant–pathogen interactions.

Keywords: fatty acid; lipid signaling; oxylipin; plant defense; lipid-transfer protein; lipase; systemic acquired resistance

10.1 Introduction

Lipids are a large group of hydrophobic molecules that are preferentially soluble in chloroform. They are major constituents of prokaryotic and eukaryotic cellular membranes and function as energy stores or signaling molecules modulating growth, development and stress response mechanisms. Plants contain a diverse array of simple and complex lipids, for example, fatty acids, phospholipids, galactolipids, sulfolipids, steroids, sphingolipids and waxes. Plastids and the endoplasmic reticulum are the two major sites for

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lipid biosynthesis in plants (Wallis and Browse, 2002). The relative insolubility of lipids in aqueous solutions and the instability of some lipid-derived molecules have constrained studies with lipids. However, the application of highly sensitive analytical technologies is rapidly advancing research on lipids and has resulted in the identification of novel lipids, some of which are present in trace amounts in plant cells (Schmelz *et al.*, 2003, 2004; Welti *et al.*, 2003; Montillet *et al.*, 2004; Welti and Wang, 2004; Andersson *et al.*, 2006a; Buseman *et al.*, 2006; Mueller *et al.*, 2006). Multiple stages of plant interactions with pathogen are influenced by host- and microbe-derived lipids. Besides the physical barrier provided by waxes on epidermal cell surface, lipids influence communication between the host and the microbe, impact pathogen development, provide elicitor and antimicrobial activities, function as signaling molecules and anchor signaling proteins to cellular membranes (Shah, 2005). Below, we review recent studies characterizing the roles of lipids, lipid-modifying enzymes and lipid-transfer proteins in plant–pathogen interactions.

10.2 Epidermal surface lipids influence plant–pathogen interactions

The epidermal surface of terrestrial plants is covered by cuticle, a hydrophobic structure that provides a water-impermeable barrier. Waxes, polysaccharides and cutin (composed mainly of C16 and C18 ω-hydroxylated esterified fatty acids) are the major constituent of the cuticular matrix (Chassot and Métraux, 2005; Nawrath, 2006). Aldehydes, fatty acids, terpenoids and phenolics are also found in the cuticle.

Products of cutin hydrolysis induce defense responses in plants. For example, cutin monomers elicited medium alkalization, ethylene production and defense gene expression when applied to cultured plant cells and effectively protected plants against fungal pathogens (Chassot and Métraux, 2005). Studies of *Arabidopsis* mutants and transgenic plants with defects in cuticle permeability provide further evidence for elicitor functions of cuticle-derived factors. Overexpression of a cutinase from *Fusarium solani* resulted in heightened resistance to *Botrytis cinerea*, although the cutinase itself did not affect fungal viability (Chassot *et al.*, 2007). Similar observations were made in the cuticle-defective *Arabidopsis bodyguard* (*bdg*) mutant and in the *bre1* (*botrytis-resistant 1*) mutant that has a defective *LACS2* gene encoding an enzyme involved in cuticle development. The *bdg* and *bre1* mutants exhibited heightened resistance to *B. cinerea* that was accompanied by increased fungitoxic activity (Bessire *et al.*, 2007; Chassot *et al.*, 2007).

In some bacteria, cutin/cutin-derived molecules repress expression of pathogenesis-associated genes. For example, compared to wild-type plants, expression of the *Pseudomonas syrinage avrPto* virulence gene and bacterial

growth were higher in the *Arabidopsis att1* (*aberrant induction of type III genes*) mutant plant in which cutin content is reduced by 70% (Xiao *et al.*, 2004). The *ATT1* gene encodes a P450 monooxygenase involved in cutin biosynthesis. Cuticular components also contribute to pathogenicity of some fungal pathogens. In *F. solani*, cutin monomers induced expression of genes encoding cutinases (Woloshuk and Kolattukudy, 1986). Similarly, in *Colletotrichum trifolii* (the causative agent of anthracnose in alfalfa), expression of a putative protein kinase C-like protein LIPK (lipid-induced protein kinase) that is involved in fungal pathogenicity was induced by cutin and its monomers (Dickman *et al.*, 2003). Appressorial tube formation in *Blumeria graminis* (syn. *Erysiphe graminis*), the powdery mildew fungus of barley, and spore germination and appressorium development in *Colletotrichum gloeosporioides* and *Magnaporthe grisea* were also promoted by cutin monomers (Podila *et al.*, 1993; Gilbert *et al.*, 1996; Tsuba *et al.*, 2002; Skamnioti and Gurr, 2007). In *M. grisea*, the *CUT2* gene, encoding a cutinase, is required for full virulence on rice. CUT2 influences the formation of the penetration peg, but does not contribute to spore or appressorium adhesion and appressorial turgor generation. Exogenously provided cutin monomers restored virulence to *cut2* mutant fungi. Moreover, virulence was restored by cAMP and diacylglycerol (DAG), suggesting that Cut2 is required for surface sensing that activates cAMP- and DAG-regulated signaling pathways involved in appressorium formation and infectious growth in *M. grisea* (Skamnioti and Gurr, 2007). Hence, although viewed primarily as a physical barrier to pathogens, the cuticle is now seen as a source of signals that influence both plant defense and microbial pathogenicity.

10.3 Elicitation of plant defenses by microbial lipids

10.3.1 Gram-negative lipopolysaccharides

Lipopolysaccharides (LPSs) present on the surface of gram-negative bacteria limit bacterial membrane permeability and thus function as a barrier to toxic compounds produced by the host. In addition, LPS contribute to pathogenicity by aiding attachment of the microbe to the host cell surface. LPS can also be perceived as pathogen-associated molecular patterns (PAMPs) eliciting defense responses in plants. For example, reactive oxygen species (ROS) generation and defense gene expression in rice were induced by LPS preparations from a variety of gram-negative bacteria (Desaki *et al.*, 2006). These responses in rice were associated with the induction of programmed cell death. Similarly, LPS preparations from *Burkholderia cepacia* when applied to *Arabidopsis* induced expression of pathogenesis-related (*PR)* genes and enhanced resistance against a virulent strain of *P. syringae* pv. *tomato* (Zeidler *et al.*, 2004). LPS-enhanced resistance was mediated through nitric oxide and

was compromised in mutant plants deficient in an enzyme affecting nitric oxide (NO) accumulation (Zeidler *et al.*, 2004). *B. cepacia* LPS also promoted the rapid phosphorylation of tobacco proteins associated with G-protein-coupled receptor signaling and Ca^{2+}/calmodulin-dependent signaling (Gerber *et al.*, 2006). Some of these responses were attenuated in the presence of staurosporine, a protein kinase inhibitor. Conversely, LPS-induced responses were intensified in the presence of calyculin A, a protein phosphatase inhibitor, suggesting the involvement of phosphorylation/dephosphorylation in addition to NO in LPS-elicited responses in plants (Gerber and Dubrey, 2004; Piater *et al.*, 2004; Gerber *et al.*, 2006). Besides eliciting defenses, LPS affect 'priming' of defense responses. Pretreatment of pepper plants with LPS resulted in the faster activation of defenses in pathogen-infected organs and enhanced resistance against *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria* (Newman *et al.*, 2000, 2002). Similar LPS priming of defenses may be associated with activation of induced systemic resistance (ISR) by root colonizing *Pseudomonas* spp. since LPS preparations from these root-colonizing bacteria were sufficient to trigger ISR in radish and carnations (Leeman *et al.*, 1995).

Studies with fluorescent-labeled LPS from *X. campestris* pv. *campestris* indicated that LPS bind to plant cell surfaces and are subsequently internalized into vacuoles via endocytosis (Gross *et al.*, 2005). The binding of labeled LPS was inhibited by unlabeled LPS and by amantadine, an inhibitor of receptor-mediated endocytosis, suggesting that this process operates through a cell surface receptor. In animals, the Toll-like surface receptor TLR4 is associated with endocytosis of LPS (Husebye *et al.*, 2006). HSP90 is another LPS receptor in animals, and both Toll-like proteins and HSP90 are also involved in plant defense responses (Shirasu and Schulze-Lefert, 2003; Nürnberger *et al.*, 2004; Sangster and Queitsch, 2005). Whether Toll-related receptors and HSP90s are important for LPS-mediated signaling in plants is not known.

LPSs have a tripartite structure composed of a lipid A moiety, a core oligosaccharaide and an O-antigen oligosaccharide (Newman *et al.*, 2007). Lipid A is the most conserved component of LPS and is a potent activator of defense responses in animals (Alexander and Rietschel, 2001). Lipid A is also an effective activator of NO synthesis in *Arabidopsis* (Zeidler *et al.*, 2004) and in reducing the severity of HR induced by *X. campestris* pv. *campestris* in pepper (Newman *et al.*, 1997). However, lipid A may not be solely responsible for all the elicitor activity of LPS; the core oligosaccharide and the O-antigen moieties also contribute to elicitor activity of LPS (Scheidle *et al.*, 2005; Newman *et al.*, 2007).

10.3.2 Fungal sphingolipids

Cerebrosides, which are sphingolipids produced by fungi, are race nonspecific elicitors of defense responses in rice. Cerebroside B produced by *Fusarium oxysporum*, the common agent of wilt disease, when applied to lettuce,

tomato, melon and sweet potato, enhanced resistance against virulent strains of *F. oxysporum* (Umemura *et al.*, 2004). Cerebrosides also enhanced resistance to downy mildew in pearl millet (Deepak *et al.*, 2003). Similarly, cerebroside A and C from *M. grisea* induced the HR, resulted in the accumulation of phytoalexins and PR proteins, and enhanced disease resistance in rice (Koga *et al.*, 1998; Umemura *et al.*, 2000, 2002). Expression of the rice *DWARF1* gene encoding the α subunit of heterotrimeric G protein was induced by cerebrosides (Suharsono *et al.*, 2002). The *d1* mutation in this rice gene resulted in the attenuation of cerebroside-induced accumulation of ROS and expression of the *PR1* gene (Suharsono *et al.*, 2002). However, ectopic expression of a constitutively active form of the small GTPase protein OsRac1 restored cerebroside-induced responses in *d1*. Co-immunoprecipitation studies indicated that OsRac1 interacts with a myelin-activated protein kinase (MAPK) OsMAPK6, which is posttranslationally activated by cerebrosides in rice suspension cells (Lieberherr *et al.*, 2005). Furthermore, the cerebroside-induced expression of *PAL* was attenuated in *OsMAPK6*-silenced rice plants. These studies suggest the involvement of a plant heterotrimeric G protein upstream of the small GTPase OsRac1 and OsMAPK6 in cerebroside signaling.

10.4 Lipid modification of defense signaling components and pathogen-derived elicitors

10.4.1 Membrane association of MAMP receptors and resistance proteins

Posttranslational addition of myristic, palmitic and phosphatidic acids as well as glycosylphosphatidylinositol (GPI) moieties can influence the activities and localizations of proteins to cellular membranes. Plasma membrane localization is critical for the function of several proteins involved in the recognition of microbe-associated molecular patterns (MAMPs) and race-specific elicitors. The bacterial elongation factor EF-Tu, flagellin-derived flg22 peptide, fungal xylanase and chitooligosaccharides which elicit defenses in a variety of plants bind the membrane-localized receptors EFR and FLS2 in *Arabidopsis*, LeEix2 in tomato and CEBiP in rice, respectively (Ron and Avni, 2004; Chinchilla *et al.*, 2006; Zipfel *et al.*, 2006; Kaku *et al.*, 2007). Similarly, perception of several race-specific elicitors is also mediated by membrane-associated R proteins and signaling components (Shah, 2005). For example, the plasma membrane-localized *Arabidopsis* RPM1 (RESISTANCE TO PSEUDOMONAS SYRINGAE pv. MACULICOLA1) and RPS2 (RESISTANCE TO PSEUDOMONAS SYRINGAE2) proteins confer race-specific resistance to *P. syringae* pathovars. RPM1- and RPS2-mediated race-specific signaling requires the NDR1 (NON RACE-SPECIFIC DISEASE RESISTANCE1) protein that is anchored to the plasma membrane by means of a GPI moiety

(Coppinger *et al.*, 2004). In animals, GPI anchors localize proteins to lipids rafts that are rich in sterols and sphingolipids (Pike, 2003). Similarly, GPI anchors may target plant proteins to lipid rafts in the cell membrane where they may aid in the recognition of pathogen-derived elicitors. Sterol-rich microdomains were formed in barley epidermal cells at sites below the attempted penetration point by *B. graminis* f.sp. *hordei*, the causative agent of powdery mildew (Bhat *et al.*, 2005). MLO1 and the ROR2 syntaxin, two barley proteins that positively and negatively regulate fungal penetration, respectively, localized to these microdomains below the fungal appressoria, suggesting that microdomains are important for interaction between barley and the powdery mildew fungus.

Computational tools have identified putative acylation sites on predicted proteins encoded by several plant resistance-associated genes (Boisson *et al.*, 2003). Trans-acylation of proteins with myristic or palmitic acid is a mechanism utilized by eukaryotes to target proteins to the inner side of the plasma membrane. In the case of *Arabidopsis* RIN4 (RPM1-INTERACTING PROTEIN 4) protein, acylation was shown to be critical for membrane localization (Mackey *et al.*, 2002). The protein acylation inhibitor 2-bromopalmitic acid compromised membrane localization of RIN4. A cluster of three Cys residues in the C-terminal part of RIN4 is required for its membrane association (Kim *et al.*, 2005). RIN4 protein also binds to RPM1 and RPS2, and cleavage of RIN4 by the bacterial effector AvrRpt2 results in activation of RPS2-dependent defense (Coaker *et al.*, 2005; Kim *et al.*, 2005). Since RPM1, RPS2, RIN4 and the corresponding pathogen-derived effectors AvrRpm1, AvrB and AvrRpt2 are targeted to plant cell membrane, this may provide a physical location to draw these molecules together. This type of indirect recognition of pathogen-derived factors by host R proteins on the cell surface may also be a mechanism for Cf2-AVR4-mediated activation of defenses in tomato against the fungal pathogen *Cladosporium fulvum* (Rooney *et al.*, 2005). Other defense-associated membrane-localized proteins have been identified that have phospholipid-binding C2, pleckstrin homology (PH) and GRAM (glucosyltransferases, Rab-like GTPase activators and myotubularins) or GPI-interacting domains that may aid in membrane association (Hua *et al.*, 2001; Vogel *et al.*, 2002; Tang *et al.*, 2005; Yang *et al.*, 2006). Thus, membrane localization may be a common theme in pathogen recognition by plant cells.

10.4.2 Modification of bacterial effector proteins by plant lipids

Bacterial pathogens secrete effector proteins into the host cell. Some of these proteins are targeted to host cell membranes where they exert their effects. For example, *Agrobacterium tumefaciens* VirE2 has a high affinity for lipid bilayers containing sterols and sphingolipids. VirE2 is involved in the formation of channels in the plasma membrane which aid in transfer of T-DNA to the host cell, suggesting that *A. tumefaciens* may utilize host lipid rafts for

pathogenicity (Duckely *et al.*, 2005). In tomato plants containing the *Pto* gene, the AvrPto effector secreted by *P. syringae* pv. *tomato*, the causative agent of bacterial speck disease, is recognized as an avirulence factor resulting in activation of defenses. *In planta*, AvrPto is myristoylated resulting in its targeting to the host cell membrane (Shan *et al.*, 2000). Mutational disruption of the myrisoylation motif in AvrPto abolished its avirulence activity (Shan *et al.*, 2000). Membrane localization was also critical for the virulence function of AvrPto (Thara *et al.*, 2004). Pto, which binds AvrPto in vivo, is also a myristoylated protein. Although not required for plasma membrane localization of Pto (Loh *et al.*, 1998), transient assays in *Nicotiana benthamiana* indicated that myristoylation of Pto is needed for AvrPto-elicited defense signaling (de Vries *et al.*, 2006).

Myristoylation/palmitoylation also targets *P. syringae* AvrB, AvrRpm1, AvrPphB and AvrRpt2 proteins to host cell membranes (Nimchuk *et al.*, 2000; Shao *et al.*, 2002; Axtell and Staskawicz, 2003). Membrane localization was shown to be critical for the virulence function of AvrRpm1 and the avirulence function of AvrRpm1 and AvrB (Nimchuk *et al.*, 2000). Myristoylation sites have also been predicted for several other effectors produced by *P. syringae* and for the YopT/AvrPphB family of effector proteins (Alfano and Collmer, 2004; Maurer-Stroh and Eisenhaber, 2004). Hence, lipid modifications may direct plant defense proteins and pathogen-derived effectors to particular sites on the cell membrane for downstream activation of defense signaling.

10.5 Signaling function of plant lipids and lipid-derived factors

10.5.1 Fatty acids

In plants, fatty acids are found predominantly in membranes conjugated to glycerol. Fatty acids also function as substrates for the synthesis of oxylipins and volatile organic compounds that are associated with indirect defenses against lepidopteran insects and regulate activity of enzymes involved in the generation of defense signaling molecules and antimicrobial compounds (Shah, 2005; Matsui, 2006). For example, polyunsaturated fatty acids (PUFAs) applied to potato and tobacco suspension cells induced the oxidative burst (Yoshioka *et al.*, 2001; Yaeno *et al.*, 2004). The effect of the trienoic fatty acid linolenic acid on an oxidative burst in tobacco cell membranes was through the activation of NADPH oxidase activity (Yaeno *et al.*, 2004). In agreement with the involvement of PUFA in the oxidative burst and plant defense, endogenous levels of linolenic acid and hexadecatrienoic acid increased rapidly in *Arabidopsis* leaves inoculated with an avirulent pathogen (Yaeno *et al.*, 2004). Furthermore, mutations in *Arabidopsis* genes encoding

ω-3 fatty acid desaturases that synthesize trienoic fatty acids resulted in the attenuation of pathogen-induced oxidative burst, confirming a role for PUFA in modulating the oxidative burst in pathogen-challenged plants. Some desaturase-like enzymes possess other novel activities. In parsley, the fungal elicitor-induced *ELI12* gene encodes a fatty acid desaturase that forms a triple bond at the Δ^{12} position, resulting in the synthesis of the Δ^{12}-acetylenic fatty acids crepenynic and dehydrocrepenynic acids (Cahoon *et al.*, 2003). Natural products derived from these fatty acids possess antimicrobial, insecticidal and nematicidal activity and are found in a variety of plants.

In contrast to PUFA, the monounsaturated fatty acid oleic acid (18:1) protected *Arabidopsis* cells from oxidative burst-associated cell death by stimulating the activity of a phospholipase, PLDδ (Zhang *et al.*, 2003). Supporting a role for monosaturated fatty acids in suppressing cell death, a reduction in oleic acid (18:1) levels in *Arabidopsis*, due to a mutation in the *SSI2* (*SUPPRESSOR OF SA-INSENSITIVITY 2*) gene encoding a desaturase that synthesizes 18:1 from 18:0 (palmitic acid), resulted in the spontaneous activation of cell death and the constitutive activation of SA-dependent defense signaling (Kachroo *et al.*, 2001; Shah *et al.*, 2001). The *ssi2* mutants exhibited heightened resistance to a variety of bacterial, viral and oomycete pathogens (Kachroo *et al.*, 2001; Shah *et al.*, 2001, 2003a; Sekine *et al.*, 2004). Reduction in 18:1 levels in the *ssi2* mutant was also associated with elevated expression of several *R* genes (Chandra-Shekara *et al.*, 2007). Expression of genes encoding 18:1 synthesizing stearoyl desaturases was upregulated in TCV-inoculated plants, suggesting that pathogens may target a plant 18:1-modulated pathway to promote disease. 18:1 fatty acids are also associated with the ability of *Arabidopsis* to turn on jasmonic acid (JA) signaling in response to pathogen infection. In the *ssi2* mutant, activation of JA-inducible defenses and resistance to the necrotrophic pathogen, *B. cinerea*, were compromised (Kachroo *et al.*, 2001; Nandi *et al.*, 2005). Exogenously provided 18:1 restored JA signaling in the *ssi2* mutant, confirming a role for 18:1 in modulating JA signaling (Kachroo *et al.*, 2001).

10.5.2 Phospholipids

Phospholipids are a large group of glycerolipids that include phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and their derivatives. The role of PA- and PI-derived inositolpolyphosphates (IPs) in plant defense signaling has been the focus of several recent studies. LPAs (lysophospholipids) and DAG are other phospholipid-derived products that are potent signaling molecules in animals. Recently, lysophosphatidylcholine was demonstrated to be a signal for the activation of phosphate transporter gene expression in arbuscular mycorrhizal colonized roots (Drissner *et al.*, 2007). LPAs and DAG are also formed in response to pathogen infection. However, their role in plant defense is poorly studied.

10.5.2.1 Phosphatidic acid

Although PA (Fig. 10.1) is a minor phospholipid in biological membranes, constituting <1% of total phospholipids, it has emerged as an important signal in plant stress responses. PA levels increase in response to stress, at times by as much as 30–65% (Wang *et al.*, 2006). Signaling PA can be released from PC, PE and PG by the action of phospholipase D (PLD). Alternatively, it can be produced by DAG kinase from DAG. It has been suggested that differences in acyl species combinations in signaling PA synthesized from these lipids can be distinguished by downstream effectors (Wang *et al.*, 2006). Transient increases in PA levels were observed after elicitor treatment of plant cells. For example, flg22, xylanase and chitin-derived elicitors induced PA accumulation in tomato suspension culture cells (Van der Luit *et al.*, 2000). PA levels also increased in response to activation of *R* gene signaling. A biphasic increase in PA was observed in leaves of *Arabidopsis* expressing AvrRpm1 or AvrRpt2 from a dexamethasone-inducible promoter (Andersson *et al.*, 2006b). Studies with radiolabeled phosphate indicated that the first burst of PA was derived from PI phosphate and the second burst from PC/PE. In contrast to the above study, PA generated in response to co-expression of the *C. fulvum* AVR4 effector and the tomato *Cf4* gene in a transgenic tobacco suspension cell system was derived primarily from PI phosphate via DAG by the sequential action of a phospholipase C (PLC) and DAG kinase (de Jong *et al.*, 2004). PA production functioned upstream of the oxidative burst in the Cf4–AVR4 system since U73122, a chemical inhibitor of a PLC activity, inhibited the AVR4-induced oxidative burst. Indeed, PA application to plant cells results in the activation of oxidative burst and cell death (Sang *et al.*, 2001; de Jong *et al.*, 2004; Park *et al.*, 2004; Andersson *et al.*, 2006b). In *Arabidopsis* leaves, PA-induced cell death was mediated by ROS produced by a Rho-related small G protein GTPase-dependent pathway (Park *et al.*, 2004). PA also has a role in plant responses to ROS. For example, PLDδ-derived PA protected *Arabidopsis* cells from H_2O_2-induced cell death (Zhang *et al.*, 2003).

Although the exact mechanism by which signaling PA impacts plant defenses is unclear, PA is known to affect vesicular trafficking and the activities of various enzymes and proteins involved in signal transduction (Testerink *et al.*, 2004; Wang *et al.*, 2006). PA binding modulates the activities of 14-3-3 transcription factors, G proteins, NADPH oxidases and HSP90, all of which are important players in plant defense against pathogens. Elevated PA concentration in membranes could also result in docking sites for protein recruitment to a membrane-associated 'signalosome', thereby enhancing particular protein–protein interactions (Munnik, 2001; Wang, 2004). In addition to affecting the biochemical function of proteins involved in signal transduction, PA serves as a precursor for the release of lysoPA, DAG and free fatty acids (Munnik, 2001; Wang, 2004), all of which might participate in plant defense signaling.

10.5.2.2 Phosphatidylinositol

IPs are potent inducers of Ca^{2+} influx in animals and plants (Stevenson *et al.*, 2000; Berridge, 2005). IPs are synthesized from PI by the actions of a series of

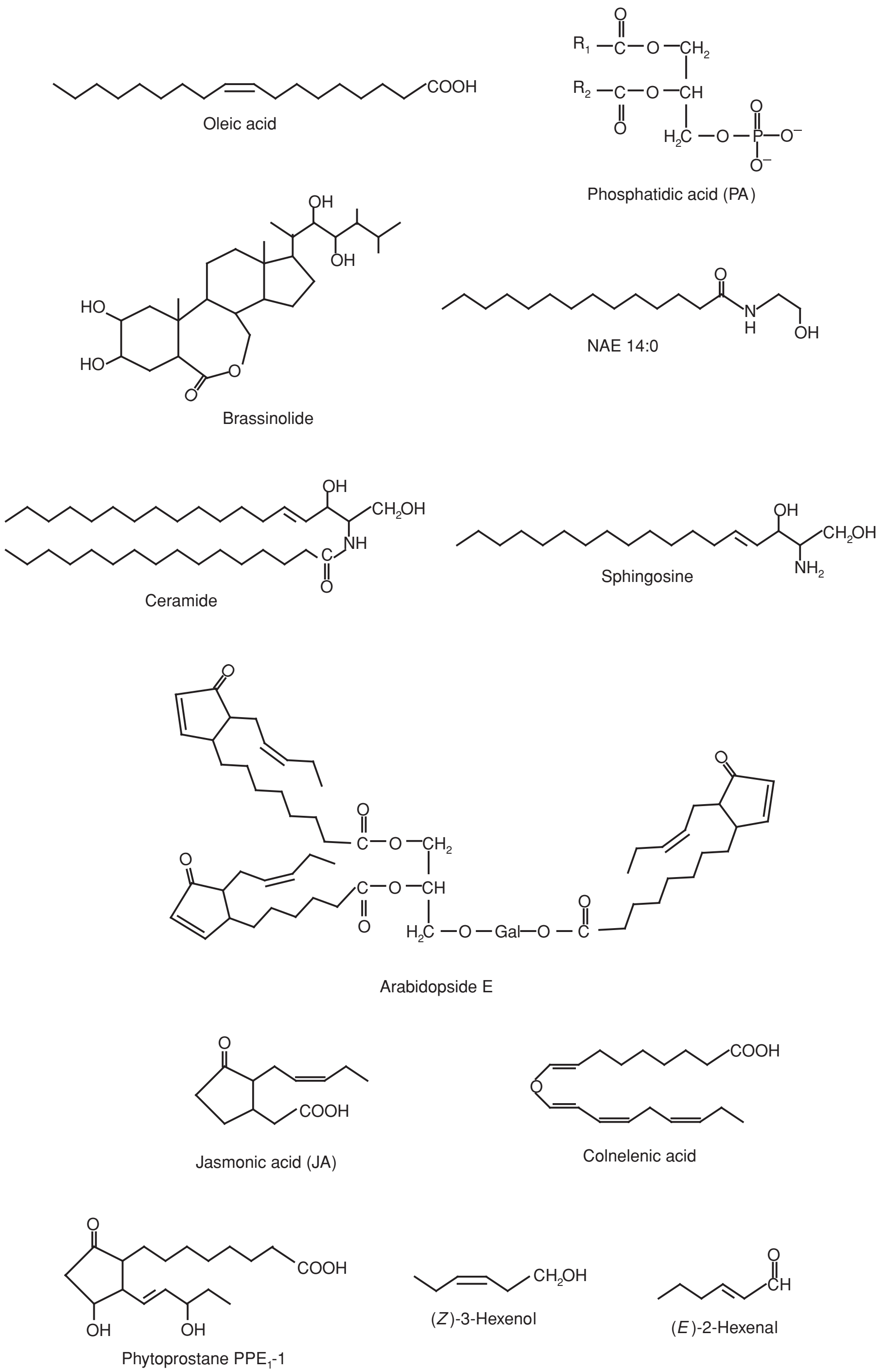

Figure 10.1 Biologically active lipids. R_1 and R_2 represent acyl chains and Gal represents galactose in phosphatidic acid and arabidopside E, respectively.

lipid kinase and, subsequently, PLC. In plants, IP accumulation is associated with the synthesis of several defense-related secondary metabolites (Vasconsuelo and Boland, 2007). A variety of microbial elicitors stimulate the accumulation of IPs. For example, chitosan induced the accumulation of inositol 1,4,5-triphosphate in the evergreen plant *Rubia tinctorum* (Vasconsuelo *et al.*, 2005). Similarly, changes in IP3 levels accompanied phytoalexin accumulation in elicitor-treated Mexican cypress cell cultures (Zhao *et al.*, 2004). Phosphoinositide turnover has also been observed in citrus infected with *Alternaria alternata* (Ortega and Perez, 2001) and in SA-treated cultured *Arabidopsis* cells (Krinke *et al.*, 2007). In *Arabidopsis* suspension cells, SA application induced a rapid increase in inositol 4-phosphate and inositol 4,5-bisphosphate levels. These changes in IP levels were attenuated by the kinase inhibitor wortmannin. Moreover, SA-induced expression of several genes was blocked by wortmannin, suggesting a link between SA signaling and phosphoinositide metabolism (Krinke *et al.*, 2007). Genetic studies in *Arabidopsis* have provided further evidence for the involvement of IPs in plant defense. Ectopic expression of a mammalian IP 5-phosphatase which hydrolyzes inositol 1,4,5-triphosphate attenuated phosphoinositide signaling in transgenic *Arabidopsis* (Im *et al.*, 2007). Basal resistance against bacterial pathogen and systemic acquired resistance (SAR) were also compromised in these transgenic plants (I. Perera, personal communication). Also, wounding-induced JA accumulation was prematurely attenuated in these lines, suggesting that inositol 1,4,5-triphosphate regulates JA accumulation (Mosblech *et al.*, 2008). IP metabolism modulates β-aminobutyric acid (BABA)-induced defense responses. For example, a mutation in the *Arabidopsis IPS2* (At*SAC1b*) gene encoding an IP phosphatase attenuated BABA-primed callose accumulation in *Arabidopsis* infected with oomycete or fungal pathogens (Ton *et al.*, 2005). BABA-induced resistance against *Hyaloperonospora parasitica* was also compromised in the *ips2* mutant plant, consistent with involvement of *IPS2* in plant resistance.

10.5.2.3 *N*-Acylethanolamines

N-Acylethanolamines (NAEs) (Fig. 10.1) are biologically active signal molecules that influence plant growth and stress responses. NAEs are derived from PE via a series of steps. It has been suggested that PLD action is involved in the release of NAE from phospholipids (Kilaru *et al.*, 2007). In contrast, NAE turnover is mediated by the action of fatty acid amide hydrolase (FAAH) yielding free fatty acid and ethanolamine, or alternatively via a lipoxygenase (LOX) pathway, resulting in the synthesis of NAE oxylipins (Kilaru *et al.*, 2007). Defense responses induced by xylanase, a fungal elicitor, are accompanied by NAE accumulation in tobacco (Tripathy *et al.*, 1999). NAE application induced expression of defense genes that are also induced by xylanase. NAE metabolism is further implicated in nonhost resistance since ectopic expression of *FAAH* compromised nonhost resistance in *Arabidopsis* (Kilaru *et al.*, 2007). Moreover, NAE metabolism affects abscisic acid (ABA) signaling (Teaster *et al.*, 2007) that has emerged as an important factor

in plant defense against pathogens (Adie *et al.*, 2007). In animals, NAE 20:4 (*N*-arachidonoylethanolamine) is an endogenous ligand for the CB-1 cannabinoid receptor found in brain cells (Hansen *et al.*, 2000). NAE-binding proteins were identified in plants (Kilaru *et al.*, 2007), but whether an NAE mode of action in plants is similar to that in animals remains to be determined.

10.5.3 Brassinosteroids

Brassinosteroids have important functions in plant growth and development but are also involved in defense against pathogens. The application of brassinolide (Fig. 10.1) protected rice against rice blast and bacterial blight disease and in tobacco enhanced resistance against *Tobacco mosaic virus* (TMV), *P. syringae* pv. *tabaci* and a fungal pathogen belonging to the genus *Oidium* (Nakashita *et al.*, 2003). Brassinolide-induced resistance in tobacco was independent of SA signaling (Nakashita *et al.*, 2003). The simultaneous activation of brassinolide- and SA-dependent defenses provided additive protection against viral and bacterial pathogens, suggesting that these are two parallel defense mechanisms. Similarly, brassinosteroid application was reported to protect potato against *Phytophthora infestans* and enhanced disease resistance in barley and cucumber plants (Krishna, 2003). Although the exact mechanism by which brassinosteroids enhance resistance in plants is unclear, brassinosteroids are known to influence a number of processes in plants, including stomatal aperture size (Haubrick *et al.*, 2006). Significantly, stomatal function was shown to regulate entry of pathogens into leaves (Melotto *et al.*, 2006) and therefore some of the effects of brassinosteroid on plant defense may be due to an impact on stomatal function.

Sterol metabolism also contributes to pathogen growth and development. Sterols are important components of eukaryotic cell membranes and some plant pathogens depend on the host for their sterol requirement (Blein *et al.*, 2002). Moreover, some fungal pathogens make toxins, for example zearalenone that resemble steroids and interferes with steroid metabolism in animals (Kiessling, 1986). Thus, fungal toxins likely also target plant steroid metabolism.

10.5.4 Sphingolipids

Sphingolipids are important components of lipid rafts that are thought to organize signaling components into microdomains of membranes (Lynch and Dunn, 2003). As mentioned above, some defense proteins (e.g. NDR1 and ROR2 syntaxin) are believed to be located in lipid rafts. Other proteins (e.g. MLO1) that contribute to susceptibility may also be located in rafts (Bhat *et al.*, 2005). Sphingolipids modulate cell death in plants, and it has been suggested that a sphingosine-1-phosphate/ceramide rheostat regulates cell death execution (Worrall *et al.*, 2003). In tomato, the application of ceramide rescued leaf cells from the lethal effects of mycotoxins (Brandwagt *et al.*, 2000). In

contrast, application of C2 ceramide induced cell death in *Arabidopsis* protoplasts (Liang *et al.*, 2003). Provision of ceramide-1-phosphate abrogated some of the effects of C2 ceramide on cell death (Liang *et al.*, 2003). Genetic evidence in *Arabidopsis* strengthens evidence for involvement of sphingolipid metabolism in modulating cell death and plant interactions with pathogens, since mutations in the *Arabidopsis ACD5* (*ACCELERATED CELL DEATH5*) and *ACD11* genes, encoding a putative ceramide kinase and sphingosine transfer protein, respectively, resulted in precocious cell death (Greenberg *et al.*, 2000; Brodersen *et al.*, 2002). In addition, the *acd11* mutant exhibited heightened resistance to bacterial pathogens. Sphingolipid metabolism and cell death are the targets of toxins produced by several necrotrophic fungi. For example, cell death that aids the necrotrophic mode of pathogenesis was induced by *Alternaria alternata* f.sp. *lycopersici* toxin and fumonisin B1, mycotoxins produced by the necrotrophic fungi *Alternaria* and *Fusarium* species, respectively. Significantly, these toxins inhibit the activity of sphinganine *N*-acyltransferase, an enzyme involved in sphingolipid metabolism (Spassieva *et al.*, 2002; Lynch and Dunn, 2003).

10.5.5 Galactolipids

Galactolipids comprise the largest group of lipids in plastid membranes and serve as reservoirs for fatty acids used in the synthesis of signaling lipids. For example, in tomato, trienoic fatty acid-containing galactolipids are believed to provide fatty acid precursors for JA synthesis in wounded leaves (Li *et al.*, 2003). In *Arabidopsis*, the *FAD7*- and *FAD8*-encoded desaturases that are involved in synthesis of trienoic fatty acid-containing galactolipids are required for plant defense against *Pythium* species (Vijayan *et al.*, 1998). Similarly, *FAD7* and the *Arabidopsis SFD1*, *SFD2* and *MGD1* genes, which are also involved in galactolipid metabolism, are needed for SAR. Such mutants are defective in the accumulation of a phloem-mobile factor required for the activation of SAR (Chaturvedi *et al.*, 2008). More recently, 200-fold increases in galactolipids containing oxylipins were reported in *Arabidopsis* leaves in response to the activation of *R* gene-mediated signaling (Andersson *et al.*, 2006a). In particular, arabidopside E (Fig. 10.1), which is a monogalactosyldiacylglycerol containing two 12-oxo-phytodienoic acids (OPDAs) and one dinor-oxo-phytodienoic acid acyl chain, accumulated to high levels (7–8% of total lipid content) in *Arabidopsis* leaves undergoing hypersensitive response (HR). A variety of such oxylipin-containing galactolipids have been identified in stressed *Arabidopsis* (Stelmach *et al.*, 2001; Hisamatsu *et al.*, 2003, 2005; Andersson *et al.*, 2006a; Buseman *et al.*, 2006). Whether these oxylipins are synthesized directly on the galactolipids or are incorporated into existing galactolipids after synthesis is not known. Also, the precise role of these oxylipins in plant defense is unclear. Synthesis of these oxylipin-esterified galactolipids could provide a mechanism for the plant to remove oxidized lipids, some of which may be toxic when present at elevated levels.

Alternatively, galactolipid-conjugated oxylipins might function as a store for the rapid release of free oxylipins upon subsequent attack by pathogen, thereby providing chemical memory. Although the exact biological relevance of oxidized galactolipids is not known, arabidopside E possesses bacteriostatic activity and arabidopside A promotes senescence, suggesting that these are biologically relevant molecules (Andersson *et al.*, 2006a; Hisamatsu *et al.*, 2006).

10.5.6 Oxylipins

Oxylipins are a large class of oxidized lipids that can be found in the free form or esterified to glycerolipids. JA (Fig. 10.1) is a prototypical oxylipin that has a signaling role in plant defense against certain pathogens and insects (Howe and Schilmiller, 2002; Farmer *et al.*, 2003; Devoto and Turner, 2005; Wasternack, 2007). Oxylipins also possess antimicrobial activities. A recent study by Prost *et al.* (2005) evaluated the antimicrobial activity of 43 naturally occurring oxylipins against a set of 13 phytopathogens. All, except two, possessed antimicrobial activity. Oxylipins can be synthesized via enzymatic and nonenzymatic routes (Fig. 10.2). For example, phytoprostanes (Fig. 10.1)

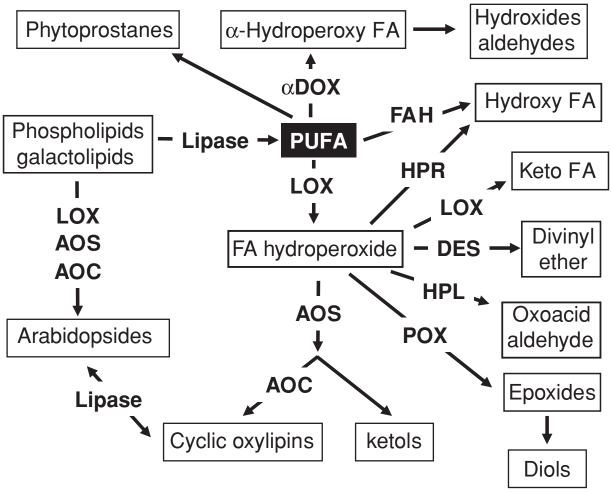

Figure 10.2 Oxylipin synthesis in plants. Oxylipin species are boxed. Phytoprostanes are synthesized from polyunsaturated fatty acid (PUFA) by nonenzymatic free-radical mechanisms. Enzymes catalyzing other reactions are as follows: AOC, allene oxide cyclase; AOS, allene oxide synthase; αDOX, α-dioxygenase; DES, divinyl ether synthase; FAH, fatty acid hydroxylase; HPL, hydroperoxide lyase; HPR, hydroperoxide reductase; LOX, lipoxygenase; POX, peroxygenase. The exact mechanism by which oxylipin esterified galactolipids (arabidopsides) are synthesized is not known; synthesis could occur through oxidation of acyl chains present on galactolipids, or alternatively, by esterification of free oxylipins to the galactolipid backbone.

are synthesized via free radical-catalyzed oxidation of PUFA. Phytoprostane levels increase in plants exposed to pathogens, presumably as a result of oxidative stress (Thoma *et al.*, 2003). Phytoprostanes applied to *Arabidopsis* cells prevented cell death triggered by toxic chemicals and induced expression of genes encoding detoxifying enzymes like glutathione S-transferases and glycosyl transferases, as well as genes involved in phytoalexin metabolism and MAPK signaling (Alméras *et al.*, 2003; Thoma *et al.*, 2003; Prost *et al.*, 2005). Constitutive accumulation of elevated phytoprostane levels correlated with elevated expression of defense genes in tocopherol-deficient *Arabidopsis* plants, further supporting their role in plant defense (Sattler *et al.*, 2006).

The first step in the enzymatic synthesis of a large number of oxylipins is catalyzed by LOXs which add molecular oxygen to PUFAs to yield fatty acid hydroperoxides (Fig. 10.2). Dienoic and trienoic fatty acids are the primary substrates for LOXs in plants. LOXs can be divided into two groups, 9-LOX and 13-LOX, on the basis of their regiospecificity. 9-LOXs synthesize 9-hydroperoxides, while 13-LOXs synthesize 13-hydroperoxides. Several LOX-derived products accumulate in plants exposed to pathogens and other elicitors of defense responses (Feussner and Wasternack, 2002; Weber, 2002; Shah, 2005). For example, in potato, 9-hydroxy octadecadienoic acid (9-HODE) accumulated at elevated levels in response to infection with *P. infestans* (Göbel *et al.*, 2001), and in barley, 13(*S*)-hydroxy octadecatrienoic acid (13-HOTrE) accumulated in SA-treated leaves (Weichert *et al.*, 1999). 13-HOTrE application induced the expression of *PR1b* in barley leaves, implicating 13-HOTrE in SA signaling. LOX-derived products are also associated with the plant HR. For example, 9-hydroxyoctadienoic and 9-hydroxyoctatrienoic acids accumulated in cryptogein-treated tobacco leaves, and restricting the accumulation of these metabolites by limiting oxygen availability resulted in the inhibition of cryptogein-induced HR (Rustérucci *et al.*, 1999). In contrast, the application of LOX-derived products to tobacco leaves induced cell death (Rustérucci *et al.*, 1999). The activation of 9-LOX was also associated with the development of HR in tobacco inoculated with *Ralstonia solanacearum* (Cacas *et al.*, 2005). Expression of *LOX* genes was reported to be induced in pathogen-infected plants and correlated with resistance. In cotton, a faster induction of a 9-LOX gene (*GhKlox1*) and 9-LOX-mediated peroxidation was seen in an incompatible interaction with *Xanthomonas campestris* compared to a compatible interaction (Jalloul *et al.*, 2002). Similarly, in *Medicago truncatula* infected with *Colletotrichum trifolii*, induction of a gene encoding a 9-LOX was faster in a resistant than in susceptible cultivar (Torregrosa *et al.*, 2004). Induction of LOX activity also correlated with systemic resistance induced by root-colonizing rhizobacteria in bean plants (Ongena *et al.*, 2004). Studies with transgenic tobacco in which expression of a gene encoding a *9-LOX* was silenced revealed an important role for 9-LOX in race-specific resistance to *P. parasitica* var. *nicotianae* and basal resistance to *Rhizoctonia solani* (Rancé *et al.*, 1998).

Recent studies with *LOX* genes suggest that oxylipins may be involved in cross-kingdom communication. Unlike the above-described defense role, some LOX-derived products appear to contribute to pathogenicity as well. 'Psi' factors produced by some fungi are structurally related to plant oxylipins (Tsitsigiannis and Keller, 2007). These fungal oxylipins regulate sporogenesis and toxin synthesis by the fungus (Brodhagen and Keller, 2006; Tsitsigiannis and Keller, 2007). In maize, loss of the *ZmLOX3* encoded 9-LOX activity resulted in heightened resistance to stalk rot caused by *Fusarium verticillioides* and *Colletotrichum graminicola* (Gao *et al.*, 2007). The *lox3* mutant plants also exhibited heightened resistance to anthracnose leaf blight caused by *C. graminicola* and the southern leaf blight pathogen *Cochliobolus heterostrophus*. Compared to wild type, conidiation and fumonisin B1 accumulation, but not vegetative growth of *F. verticillioides*, were reduced in the kernels of *lox3* mutants. Conidiation and toxin production by *C. graminicola* were also reduced in the *lox3* mutant plants, suggesting that maize LOX3-derived products are required generally for fungal pathogenesis and reproduction. Indeed, in peanut, 9-LOX-derived product(s) promoted sporulation and mycotoxin production by *Aspergillus* (Burow *et al.*, 1997, 2000; Tsitsigiannis *et al.*, 2005).

Products of LOX-catalyzed reactions are the substrates for several enzymes involved in the synthesis of a large number of aldehydes, divinyl ethers, jasmonates and a blend of specific volatiles (Feussner and Wasternack, 2002; Weber, 2002; Shah, 2005). For example, the channeling of 18:3-hydroperoxide to allene oxide synthase (AOS) results in the synthesis of OPDA, which can then be converted in JA. Epoxy- or dihydrodiol polyenoic fatty acids are synthesized by the action of peroxygenases on hydroperoxides. In contrast, divinyl ether synthase transforms fatty acid hydroperoxides into colneleic and colnelenic acids (Weber *et al.*, 1999; La Camera *et al.*, 2004). Colneleic and colnelenic acids accumulate in tobacco leaves inoculated with TMV and *Phytophthora parasitica* var. *nicotianae* race 0 (Weber *et al.*, 1999; Fammartino *et al.*, 2007). In potato plants inoculated with *P. infestans*, colneleic and colnelenic acids accumulated more rapidly in the moderately resistant cultivar Matilda than in the susceptible cultivar Bintje (Weber *et al.*, 1999), suggesting a role for these divinyl ethers in resistance to late blight. Indeed, colneleic and colnelenic acids inhibit germination of *Phytophthora* zoospores (Fammartino *et al.*, 2007), reinforcing a role for these lipids in plant defense against *Phytophthora*.

Hydroperoxide lyases (HPLs) action leads to the release of short-chain aldehydes and alcohols and their oxoacid, traumatic acid, from hydroperoxides. Some of these aldehydes and alcohols possess antimicrobial activities. For example, *Aspergillus flavus* growth in corn was inhibited by (*E*)-2-hexenal (Zeringue *et al.*, 1996), and *P. syringae* growth in lima bean leaves was inhibited by (*E*)-2-hexenal and (*Z*)-3-hexenol (Croft *et al.*, 1993). Furthermore, elevated expression of HPL and (*Z*)-3-hexenal accumulation correlated with the activation of ISR in bean plants (Ongena *et al.*, 2004).

In addition to LOXs, the oxidation of PUFA can be catalyzed by α-dioxygenase (αDOX) and cytochrome P450-type fatty acid hydroxylases

(Blée, 2002; Feussner and Wasternack, 2002; La Camera *et al.*, 2004). αDOXs exhibit homology to prostaglandin endoperoxide synthases. Tobacco αDOX was identified as a pathogen-inducible dioxygenase and catalyzes the formation of unstable 2(*R*)-hydroperoxy fatty acids (Sanz *et al.*, 1998; Hamberg *et al.*, 1999). These unstable products can subsequently be reduced to the corresponding alcohol or decarboxylated into the next lower fatty aldehyde (Hamberg, 1999). Levels of αDOX-derived 2-hydroxylinolenic acid and 8,11,14-heptadecatrienoic acid increased during an incompatible interaction between tobacco and *P. syringae* pv. *syringae* (Hamberg *et al.*, 2003). The extent of necrosis caused by the pathogen was markedly reduced when bacteria were co-infiltrated with 2-hydroxylinolenic acid, suggesting a protective effect of this αDOX-derived lipid. Similarly, reduced expression of the *αDOX1* gene in *Arabidopsis* resulted in increased susceptibility to an avirulent *P. syringae* strain that was accompanied by a more rapid and severe necrotic response (de León *et al.*, 2002). Pathogen-induced expression of *αDOX1* was confined to the infection site in an incompatible interaction, suggesting that αDOX1 is involved in protecting plants against oxidative stress-induced cell death (de León *et al.*, 2002).

10.5.6.1 Jasmonates

Jasmonates are a large group of cyclicized oxylipins that include JA and its derivatives. JA is the best-studied signaling oxylipin in plant stress response. Readers are directed to several reviews that have summarized JA biosynthesis, signaling and role in plant defense (Howe and Schilmiller, 2002; Farmer *et al.*, 2003; Devoto and Turner, 2005; Lorenzo and Solano, 2005; Fujita *et al.*, 2006; Wasternack, 2007). Our coverage of JA will be restricted to very recent developments in JA signaling, especially those pertaining to plant disease resistance.

A recent study (Truman *et al.*, 2007) points to a role for JA in long-distance signaling associated with SAR. In *Arabidopsis*, SAR can be induced in the distal leaves by prior exposure of other leaves to an avirulent pathogen. The activation of SAR results in heightened resistance to subsequent challenge by pathogens. Truman *et al.* (2007) observed that avirulent strains of *P. syringae* when infiltrated into *Arabidopsis* leaves resulted in elevated expression of jasmonate biosynthetic genes around the vasculature. Also, within 5 h of infiltration of the avirulent pathogen, expression of several JA-responsive genes, including *JIN1* (*JASMONATE INSENSITIVE1*) and *VSP2* (*VEGETATIVE STORAGE PROTEIN2*), were elevated in uninoculated leaves. Furthermore, in comparison to wild type, SAR-conferred disease resistance against *P. syringae* was compromised in distal leaves of the JA-deficient *opr3* and JA-insensitive *jin1* and *jai4* mutants. However, since SAR is not compromised in the JA-insensitive *coi1* and *jar1* mutants (Cui *et al.*, 2005; Mishina and Zeier, 2007), this involvement of JA in long-distance signaling associated with SAR, as suggested by Truman *et al.* (2007), must be independent of *COI1* and *JAR1*. Differences in dose of the avirulent pathogen used to activate SAR in these

studies could also contribute to discrepancies in the requirement of these JA signaling genes in SAR. Cui *et al.* (2005) and Mishina and Zeier (2007) used avirulent pathogens at a density of 10^7–10^8 CFU/mL for activating SAR. At these pathogen doses, HR occurs in the avirulent pathogen-inoculated leaf within 24 h. In contrast, HR does not develop if the pathogen concentration is below a certain threshold (Turner and Novacky, 1974). Although not essential, HR potentiates the activation of SAR so that SAR is weaker in the absence of hypersensitive plant cell death (Cameron *et al.*, 1994). In their study, Truman *et al.* (2007) used a non-HR-inducing dose (10^5 CFU/mL) of the avirulent *P. syringae* strain to trigger SAR. Other studies have shown that the *jin1* and *opr3* exhibit heightened basal resistance to *P. syringae* (Nickstadt *et al.*, 2004; Laurie-Berry *et al.*, 2006; Raacke *et al.*, 2006). Moreover, *JIN1*, although a positive regulator of JA-mediated wound responses, is a negative regulator of JA signaling in plant defense (Lorenzo *et al.*, 2004). Reduced growth of the SAR eliciting *P. syringae* strain in the *jin1* and *opr3* mutants, relative to the wild-type plant, could compromise the synthesis/accumulation of the long-distance SAR signal, thus accounting for the attenuation of SAR in the *jin1* and *opr3* mutants as reported by Truman *et al.* (2007). Indeed, when an HR-inducing dose (10^7 CFU/mL) of an avirulent *P. syringae* strain was used, the *opr3* mutant plant was SAR competent (K. Krothapalli and J. Shah, unpublished data). Furthermore, although jasmonate levels do increase in petiole exudates of avirulent pathogen-infiltrated leaves, JA and methyl-JA (MeJA) do not copurify with the SAR-inducing activity, and when infiltrated into *Arabidopsis* leaves, JA and MeJA did not promote SAR in distal leaves (Chaturvedi *et al.*, 2008), suggesting that JA/MeJA by themselves are not sufficient to promote long-distance signaling associated with SAR. However, JA may still be required in the distal leaf, subsequent to the perception of the long-distance SAR signal.

The *Arabidopsis CORONATINE INSENSITIVE1* (*COI1*) gene is required for JA-mediated responses. COI1 is an F-box protein that functions in the Skp, Cullin, F-box containing complex ubiquitin–proteosome pathway (Xu *et al.*, 2002). Similarly, the AXR1 (AUXIN RESISTANT1) and SGT1b/JAI4 (JASMONATE INSENSITIVE4) proteins, two other components of the ubiquitin–proteosome pathway, are involved in JA signaling (Lorenzo and Solano, 2005). Defense against necrotrophic fungi was compromised in the *coi1* mutant plant (Glazebrook, 2005). Similarly, disease resistance was compromised in the *axr1* and *sgt1b* mutant plants (Austin *et al.*, 2002; Azevedo *et al.*, 2002; Tiryaki and Staswick, 2002), suggesting an important role for the ubiquitin–proteosome pathway in the turnover of a negative regulator of JA signaling in plant defense. Until recently, the identity of such negative regulator(s) of JA signaling had been elusive. The *jasmonate insensitive3* (*jai3-1*) mutant allele exhibits a dominant-negative JA-insensitive phenotype, suggesting that the JAI3 protein is a negative regulator of JA signaling (Lorenzo *et al.*, 2004). The JAI3 protein was recently shown to interact physically with COI1, and jasmonate treatment resulted in the COI1-dependent turnover

of JAI3 (Chini *et al.*, 2007). By contrast, the *jai3-1*-encoded truncated JAI1-3 protein was resistant to degradation by the ubiquitin–proteosome pathway and ectopic expression of JAI1-3 phenocopied the *jai1-3* mutant phenotype (Chini *et al.*, 2007). These results indicate that JAI3 turnover by the ubiquitin–proteosome machinery is relevant to its role in JA signaling. JAI3 belongs to the JAZ (jasmonate-ZIM (zinc-finger protein expressed in inflorescence meristem) domain) family of proteins (Chini *et al.*, 2007; Thines *et al.*, 2007). As with JAI3 (and JAZ3), JAZ1 protein also physically interacted with COI1 and a dominant-negative *35S-JAZ1Δ3* chimera conferred jasmonate insensitivity in transgenic *Arabidopsis* (Thines *et al.*, 2007). Furthermore, the *35S-JAZ1Δ3* transgenic plants exhibited a disease resistance phenotype that was similar to JA-insensitive *coi1* plants (Thines *et al.*, 2007), confirming a role for JAZ proteins in plant defense and JA signaling. JAI3 also binds to the transcription factor AtMYC2 encoded by the *JIN1/JAI1* (*JASMONATE INSENSITIVE 1*) gene, which modulates JA signaling and disease resistance in *Arabidopsis* (Devoto and Turner, 2005; Lorenzo and Solano, 2005; Fujita *et al.*, 2006; Wasternack, 2007). AtMYC2 antagonizes expression of some JA-responsive genes (*PDF1.2* and *PR4*), and in agreement with a negative role for AtMYC2 in *Arabidopsis* defense against pathogens, the *jin1/jai1* mutant plant exhibited enhanced resistance to *P. syringae*, *Plectosphaerella cucumerina*, *B. cinerea* and *F. oxysporum* (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004; Nickstadt *et al.*, 2004; Laurie-Berry *et al.* 2006). Strikingly, AtMYC2 promotes another arm of JA signaling leading to activation of the JA-responsive genes *VSP2* and *LOX3* (Lorenzo *et al.*, 2004), suggesting that AtMYC2's impact on JA signaling is influenced by additional factors. ERF1 (ETHYLENE RESPONSE FACTOR1) is one such factor needed for induction of JA signaling in defense against necrotrophic pathogens, but suppressing JA signaling in the wound response (Berrocal-Lobo *et al.*, 2002; Lorenzo *et al.*, 2003). These results accord with previous observations that ethylene antagonizes certain JA-mediated responses (Rojo *et al.*, 2003), although ethylene and JA can also function cooperatively to promote other defenses (Bostock, 2005; Broekaert *et al.*, 2006). A JA-inducible MPK6-dependent MAPK cascade modulates the *JIN1*-regulated pathway (Takahashi *et al.*, 2007), and a loss-of *mpk6* activity compromised JA-induced accumulation of *PDF1.2* transcripts but increased *VSP2* expression compared to wild type.

The complex nature of AtMYC2's function in plant defense might relate to its involvement in ABA signaling. AtMYC2 binds cis-elements in the promoter of dehydration-induced genes, and the *jin1* mutant is also less sensitive to ABA (Fujita *et al.*, 2006). The ABA-deficient *aba2* mutant is also more resistant to the fungal pathogen *F. oxysporum* (Anderson *et al.*, 2004). Supporting an antagonistic interaction between JA and ABA, high concentrations of ABA-suppressed expression of JA inducible genes and the ABA-deficient *aba1* and *aba2* mutants exhibited heightened expression of JA-responsive genes (Anderson *et al.*, 2004). Similarly, an antagonistic interaction between JA and ABA signaling was also observed in the *jar1* mutant that is hypersensitive to ABA

(Staswick *et al.*, 1992). In contrast, the *BOS1* (*BOTRYTIS SUSCEPTIBLE1*) gene encoding a protein with homology to the Myb family of transcription factors positively regulates both ABA and JA signaling in *Arabidopsis* (Mengiste *et al.*, 2003). *BOS1* regulates expression of a subset of JA- and ABA-inducible genes in plant stress responses. The loss of *BOS1* function confers enhanced susceptibility to *B. cinerea* and *Alternaria brassicicola* and reduced tolerance to salinity and drought. Notably, a recent study revealed a link between stomatal function and innate immunity competence in plants (Melotto *et al.*, 2006). However, the role of ABA in this response is not known.

AXR1 is a positive regulator of the SCF^{TIR1} ubiquitin–proteosome pathway that modulates auxin signaling. Inclusion of AXR1 as a component of the SCF^{COI1} signalosome indicates that JA and auxin signaling share common components, and genetic studies confirm the existence of cross talk between JA and auxin signaling. The *axr1* mutant has a JA-insensitive phenotype in addition to resistance to auxins (Tiryaki and Staswick, 2002). In addition, *axr1* exhibited heightened susceptibility to the opportunistic pathogen *Pythium irregulare* (Tiryaki and Staswick, 2002). In striking contrast, auxin and JA signaling (see below) negatively affect plant defense against the bacterial pathogen *P. syringae*. Repression of auxin signaling by a microRNA that targets expression of the *TIR1* gene encoding the F-box TIR1 protein that is a component of the SCF^{TIR1} ubiquitin–proteosome pathway, enhanced *Arabidopsis* resistance against *P. syringae* (Navarro *et al.*, 2006). Expression of the *Arabidopsis LOX2* and *AOS* genes which are involved in JA synthesis, and the JA-responsive *VSP* gene, is induced by auxin application, and stimulation of these genes by auxin is dependent on *AXR1* function (Tiryaki and Staswick, 2002). By contrast, MeJA-induced expression of *VSP* is only marginally affected in the *axr1* mutant. Since, JA synthesis is under control of a positive feedback loop, these results suggest that auxin may influence JA synthesis by modulating this positive feedback loop. The involvement of auxin in plant defense was recently reviewed by Seilaniantz *et al.* (2007).

In general, JA has a more profound impact on resistance against necrotrophic pathogens. In contrast, SA has a major role in resistance against biotrophs and hemibiotrophs. Cross talk between these two pathways is important in fine-tuning the overall defense output. The synergistic and antagonistic interplay between JA and SA signaling has been extensively reviewed (Bostock, 2005; DeVoto and Turner, 2005; Shah, 2005; Chaturvedi and Shah, 2007). Oleic acid modulates antagonistic cross talk between these defense signaling mechanisms by promoting JA signaling and negatively impinging upon SA signaling (Kachroo *et al.*, 2001, 2003a; Shah, 2005). As discussed above, reduction in oleic acid levels in the *ssi2* mutant was accompanied by an inability of JA to induce *PDF1.2* expression and heightened susceptibility to the necrotroph *B. cinerea*. By contrast, the *ssi2* mutant constitutively accumulated elevated SA and exhibited hyperactivation of SA signaling leading to heightened resistance against *P. syringae* and *H. parasitica* (Kachroo *et al.*, 2001; Shah *et al.*, 2001, 2003a). Suppressor mutations in which oleic acid level

were restored resulted in reversal of the *ssi2*-conferred phenotypes (Kachroo *et al.*, 2003b; Nandi *et al.*, 2003). Furthermore, oleic acid application restored JA responsiveness in the *ssi2* leaves (Kachroo *et al.*, 2003a), confirming the involvement of oleic acid in induction of JA-mediated responses. During the last decade, several genes have been proposed to mediate JA–SA pathway cross talk. For example, the *Arabidopsis* MPK4 MAP kinase, which is required for JA signaling, antagonizes SA signaling, suggesting involvement of MAP kinase cascade in this process (Petersen *et al.*, 2000). The *NPR1* gene, by contrast, is required for the SA response and represses JA signaling. However, different pools of NPR1 may affect the SA and JA pathways. SA signaling requires nuclear localized NPR1 whereas cytosolic NPR1 is necessary to antagonize JA signaling (Durrant and Dong, 2004). The WRKY70 DNA-binding protein may be important for mediating this cross talk (Li *et al.*, 2004). Constitutive overexpression of *WRKY70* enhanced expression of SA-inducible *PR* genes and resistance against *P. syringae* and *Erwinia carotovora* but suppressed the MeJA-induced expression of *PDF1.2*. Conversely, silencing of *WRKY70* enhanced basal expression of JA-responsive genes, consistent with an inhibitory effect of WRKY70 on the JA response. The *WRKY70* status did not affect SA and JA accumulation, suggesting that it functions downstream of these signaling molecules. Indeed, expression of *WRKY70* was induced by SA and repressed by JA application. WRKY33 is another member of the WRKY transcription factor family that modulates cross talk between SA and JA signaling pathways in plant disease resistance. However, unlike *WRKY70*, overexpression of *WRKY33* compromised resistance to *P. syringae* that was associated with a slower induction of SA-responsive genes (Zheng *et al.*, 2006). Accordingly, resistance to the necrotrophic fungal pathogens *B. cinerea* and *A. brassicicola* was enhanced in *WRKY33* overexpresser lines and compromised in *wrky33* mutant plants (Zheng *et al.*, 2006). Pathogen-infection-induced expression of the JA-responsive *PDF1.2* gene was also compromised in the *wrky33* mutant. *WRKY33* expression is induced by pathogen infection. However, JA signaling is not required for its induction, but rather *COI1* negatively affects the pathogen-induced accumulation of *WRKY33* mRNAs while SA induces *WRKY33* transcript accumulation (Zheng *et al.*, 2006), suggesting tight feedback regulation of *WRKY33* expression by SA and JA.

10.6 Lipases in plant–pathogen interaction

10.6.1 Phospholipases

Phospholipases catalyze the hydrolysis of phospholipids and, depending on the linkage they hydrolyze, can be classified as PLA, PLC and PLD (Fig. 10.3). All three groups of phospholipases have been implicated in plant–pathogen interactions (Wang, 2001, 2004; Laxalt and Munnik, 2002; Shah, 2005; Wang

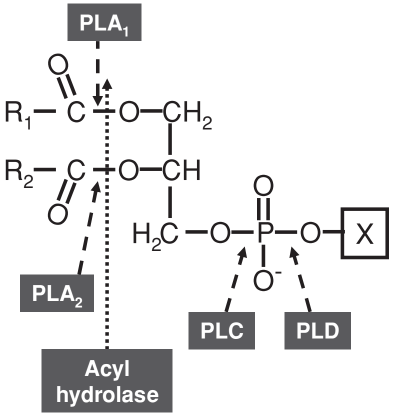

Figure 10.3 Phospholipid backbone showing sites of action of lipases. PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; X, head group: H in phosphatidic acid, choline in phosphatidylcholine, ethanolamine in phosphatidylethanolamine, inositol in phosphatidylinositol, and glycerol in phosphatidylglycerol.

et al., 2006). These phospholipases generate a variety of signaling molecules including fatty acids, PA, LPA, DAG and IPs.

PLA action on phospholipids releases free fatty acids and LPA. Pathogen infection affects PLA activity and gene expression. In *Arabidopsis*, for example, expression of a gene encoding a putative PLA IIA enzyme is induced in response to inoculation with the fungal pathogens *A. alternata*, *A. brassicicola* and *Colletotrichum higginsianum* (Narusaka *et al.*, 2003). Similarly, in tobacco, expression of three genes encoding patatin-like enzymes that display high PLA activity were induced by TMV infection (Dhondt *et al.*, 2000). Furthermore, *NtPat*-encoded PLA_2 activity was induced in tobacco leaves inoculated with the necrotrophic pathogens *E. carotovora* and *B. cinerea* or in response to treatment with the cell death-inducing proteinaceous elicitor β-megaspermin (Dhondt *et al.*, 2002). In the β-megaspermin-infiltrated leaves, activation of PLA_2 correlated with the accumulation of JA and its precursor OPDA. Both PLA_2 activity and OPDA/JA accumulated to elevated levels in the elicitor-infiltrated zones well before the onset of cell death. Relatively little PLA_2 activity and OPDA/JA were found outside the infiltrated area. It is likely that PLA_2 activity contributes to the release of fatty acids for oxylipin synthesis in tissues undergoing cell death, akin to the involvement of the *Arabidopsis DEFECTIVE IN ANTHER DEHICENCE 1* (*DAD1*)-encoded PLA in JA synthesis in floral organs (Ishiguro *et al.*, 2001). Ergosterol, a fungal lipid, also specifically induced PLA_2 activity in tobacco suspension cells (Kasparovsky *et al.*, 2004). Ergosterol-induced ROS burst and alkalization of the culture medium were blocked by arachidonic acid trifluoromethyl ketone, a specific inhibitor of PLA_2 activity, confirming a role for PLA_2 in mediating plant responses to

ergosterol. Alterations in PLA_2 activity have also been reported in glycoprotein elicitor-treated parsley and California poppy suspension cells (Scherer *et al.*, 2002; Viehweger *et al.*, 2002). The activation of PLA_2 was accompanied by rapid intracellular acidification (Viehweger *et al.*, 2002). Intracellular acidification could also be induced by the application of lysophosphatidylcholine, a product of PLA_2 action, suggesting that in the suspension cells PLA_2-derived LPAs may influence early responses to microbial elicitors.

The action of PLC on phospholipids generates the signaling molecules IPs and DAG, and as mentioned above, IPs are implicated in defense signaling. It is likely that a PI-specific PLC is responsible for the release of such IP signals. In animals, DAG is a potent activator of protein kinase C. Plants do not have a known protein kinase C activity. However, in plants, DAG can be converted into signaling PA by DAG kinase (Wang *et al.*, 2006). Indeed, a PLC activity was demonstrated to be involved in the transient accumulation of PA during the incompatible interactions between the tomato *R* gene *Cf4* and the *C. fulvum* AVR4 elicitor (de Jong *et al.*, 2004). More recently, Andersson *et al.* (2006b) reported that PA accumulates in two phases during incompatible interactions in *Arabidopsis* using a pathogen-free system in which dexamethasone-inducible *P. syrinage* AvrRpm1 and AvrRpt2 avirulence factors were transiently expressed. Using inhibitors of PI-PLC, they provided evidence that PI-PLC contributes to the first burst of PA. Further, they suggest that IPs produced by PLC may stimulate Ca^{2+} fluxes in extracellular spaces that could activate DAG kinase, resulting in the synthesis of PA from DAG to produce the first burst of PA in this system. PLC activity is also required for the ROS burst associated with the incompatible interaction between the tomato *R* gene *Cf4* and the *C. fulvum* AVR4 elicitor (de Jong *et al.*, 2004) and in rice suspension cells treated with *N*-acetylchitooligosaccharide, a chitin-derived elicitor (Yamaguchi *et al.*, 2003).

In the *Arabidopsis* incompatible interaction involving the AvrRpm1 and AvrRpt2 avirulence factors, the second burst of PA resulted from PLD action on PC and PE (Andersson *et al.*, 2006b). Furthermore, infiltration of PLD into *Arabidopsis* leaves induced HR-like lesions and expression of a *PR* gene (Andersson *et al.*, 2006b). 1-Propanol, an inhibitor of PLD, lowered PA accumulation by 60% and reduced the HR-associated ion leakage. Experiments with diphenyleneiodonium, an inhibitor of NADPH oxidase, indicated that PA functions upstream of ROS production in this incompatible interaction in *Arabidopsis*. Similarly, in rice suspension cells treated with *N*-acetylchitooligosaccharide, two PA bursts were observed and the second PA burst was dependent on PLD activity (Yamaguchi *et al.*, 2005). Induction of PLD activity has also been reported in rice leaves infected with *Xanthomonas oryzae* pv. *oryzae*, and the enzyme was recruited to sites on plasma membrane adjacent to the pathogen (Young *et al.*, 1996). Similarly, PLD activity was induced in tomato suspension cells treated with elicitors (Van der Luit *et al.*, 2000). In some instances, pathogen infection and elicitor treatment also result in the induction of *PLD* expression. For example, expression of several

Arabidopsis PLD genes was enhanced in response to infection with *P. syringae* (de Torres Zabela *et al.*, 2002). Also, in tomato, expression of *LePLDβ1* was upregulated after xylanase treatment (Laxalt *et al.*, 2001). Silencing of *LePLDβ1* expression in tomato suspension cells resulted in hyperaccumulation of H_2O_2 in response to xylanase treatment (Bargmann *et al.*, 2006), suggesting that PLDδ may protect against H_2O_2. Indeed, in *Arabidopsis*, an oleic acid-responsive PLDδ activity antagonized H_2O_2-induced cell death (Zhang *et al.*, 2003). Thus, different PLDs may have contrasting roles in promoting or antagonizing the effects of ROS, presumably due to different signaling PAs they generate.

10.6.2 Other lipases

Several nonspecific acyl hydrolases also contribute to plant–pathogen interaction. The involvement in plant defense of some genes encoding proteins with homology to acyl hydrolases was recently reviewed (Shah, 2005). More recently, the *Arabidopsis* AtPLAI lipase, which can hydrolyze phospholipids as well as galactolipids, was demonstrated to have an important role in defense against the necrotrophic fungus *B. cinerea* (Yang *et al.*, 2007). Leaves of the *Atpla1* mutant were more severely damaged by *B. cinerea* than the wild-type plants. Also, in comparison to wild type, basal levels of JA were lower in *Atpla1* mutants (Yang *et al.*, 2007). However, pathogen-induced levels of JA were unaffected in the mutant. MeJA application alleviated the disease severity phenotype of mutants, suggesting that AtPLAI-dependent basal JA level contributes to defense against *B. cinerea*. GLIP1 is another lipase in *Arabidopsis* that is required for resistance against necrotrophic pathogen (Oh *et al.*, 2005). GLIP1 protein inhibits germination of *A. brassicicola* spores, causing severe changes in spore morphology. Mutations in the active site of GLIP1 that eliminate lipase activity also resulted in loss of antifungal activity, thus confirming that lipase activity is required for GLIP1s antifungal activity. The application of GLIP1 to *Arabidopsis* also induced systemic resistance against *A. brassicicola* (Oh *et al.*, 2005), suggesting that it may also stimulate systemic defense signaling.

Lipases also contribute to plant susceptibility. For example, silencing the expression of the *Arabidopsis PLP2* gene that encodes a patatin-like acyl hydrolase resulted in enhanced resistance to *B. cinerea* and an avirulent strain of *P. syrinage* (La Camera *et al.*, 2005). In contrast, plants overexpressing *PLP2* exhibited heightened sensitivity to *B. cinerea* and avirulent *P. syringae*, and *PLP2* expression levels correlated with the extent of cell death in pathogen-inoculated plants. SOBER1 (SUPPRESSOR OF AVRBST-ELICITED RESISTANCE1) is another putative lipase that contributes to susceptibility in *Arabidopsis*. The absence of a functional *SOBER1* gene in accession Pi-0 confers resistance to *P. syringae* engineered to express AvrBsT1, a type III effector from *X. campestris* pv. *vesicatoria* (Cunnac *et al.*, 2007). This resistance was dependent on the defense component *NDR1*

and SA signaling, suggesting that SOBER1 modulates activation of gene-for-gene resistance (Cunnac *et al.*, 2007). A SOBER1-derived factor could function in inhibiting elicitation of defenses by AvrBsT1. Alternatively, SOBER1 may be a more direct susceptibility factor in *Arabidopsis*. Indeed, several fungi are known to depend on lipid-derived cues from the host to complete their life cycles (Tsitsigiannis and Keller, 2007). Others require host lipid-derived cues for pathogenicity. For example, *Fusarium graminearum*, the principal agent of *Fusarium* head blight disease in wheat and barley, produces an extracellular lipase, FGL1 (Voigt *et al.*, 2005). Expression of *FGL1* is induced *in planta* or in culture medium supplemented with wheat germ oil. On synthetic medium, a mutation in *FGL1* had no detrimental effect on fungal growth. However, *fgl1* mutant fungi were unable to spread from the inoculated wheat floret through rest of the spike (Voigt *et al.*, 2005), consistent with an *FGL1*-encoded lipase being required for pathogenicity.

10.7 Lipid-transfer proteins in plant–pathogen interaction

Lipid-transfer proteins (LTPs) are small proteins that have the ability to bind lipids and other hydrophobic molecules. Most LTPs have an α-helical structure that is stabilized by four disulfide bonds involving eight cysteine residues, resulting in a hydrophobic tunnel/cavity that allows binding of the ligand (Cheng *et al.*, 2004; Marion *et al.*, 2007). LTPs bind a variety of lipids, for example, fatty acids, phospholipids, sterols and oxylipins. Plant LTPs can be classified into two groups, LTP1 and LTP2, both of which possess a signal peptide sequence that targets these proteins to apoplast. Readers are directed to a recent review for additional information on the structure and function of plant LTPs (Carvalho and Gomes, 2007). LTPs serve a variety of direct and indirect functions in plant defense. For example, LTPs shuttle lipid precursors for the synthesis of cuticle on the cell surface. As discussed below, plant LTPs have been implicated in defense signaling and LTPs produced by pathogens also influence plant–pathogen interactions.

In pepper, three LTPs (CaLTPs) are expressed at elevated levels in *X. campestris* pv. *vesicatoria*-infected leaves (Jung *et al.*, 2003). Expression of CaLTPs was also induced systemically after local pathogen inoculation. In situ hybridization indicated that *CaLTP1* expression localized to the phloem (Jung *et al.*, 2003). Constitutive overexpression of the *CaLTP1* cDNA in transgenic *Arabidopsis* resulted in heightened resistance against *P. syringae* pv. *tomato* and *B. cinerea* (Jung *et al.*, 2005). Similarly, overexpression of three LTPs encoded by the *Arabidopsis* At4g12470, At4g12480 and At4g12490 genes enhanced resistance against *B. cinerea* (Chassot *et al.*, 2007). High-level expression of these genes also correlated with heightened resistance to *B. cinerea* in transgenic *Arabidopsis* expressing a fungal cutinase, suggesting that cutin

monomers may be involved in the induction of certain LTP-encoding genes (Chassot *et al.*, 2007).

Some LTPs have antimicrobial activities that could directly contribute to their role in plant defense (Carvalho and Gomes, 2007). For example, puroindolines, LTPs found in seeds of Triticae and Avenae tribes, possess antimicrobial properties against fungal pathogens (Marion *et al.*, 2007). In addition, puroindolines enhance the effect of antimicrobial peptides such as defensin. Similarly, HA-AP10 LTP from sunflower (*Helianthus annuus*) seeds was found to have a fungistatic effect, blocking germination of *F. solani* f.sp. *eumartii* spores (Regente and de la Canal, 2000). Although the exact mechanism by which LTPs inhibit microbial growth is not known, it has been suggested that LTPs may bind and permeabilize membranes, thereby contributing to their antimicrobial activity (Diz *et al.*, 2006). The ability of LTPs to adduct oxylipins (Bakan *et al.*, 2006) may also contribute to host defense by limiting the availability of these lipids to the pathogen. As discussed above, 9-LOX-derived oxylipins are required by some fungal pathogens for growth and development (Tsitsigiannis and Keller, 2007). Similarly, as discussed below, some oomycete pathogens also depend on their hosts for essential lipids.

Phytophthora and *Pythium* spp. are unable to produce sterols needed for asexual and sexual reproduction. These oomycetes produce elicitins, a class of LTPs that shuttle plant synthesized sterols to the pathogen (Panabières *et al.*, 1997; Blein *et al.*, 2002; Kamoun, 2006). β-Cryptogein is an elicitin produced by *Phytophthora* that captures essential ergosterol (Osman *et al.*, 2001a,b; Cheng *et al.*, 2004). However, plants such as tobacco have evolved mechanisms to recognize the elicitin/sterol complex by means of cell surface receptors, resulting in activation of phytoalexin accumulation, expression of *PR* genes and HR-like cell death (Blein *et al.*, 2002; Kamoun, 2006). Some of the early physiological changes induced by elicitin include Ca^{2+} fluxes, media alkalization, ROS accumulation and lipid peroxidation (Blein *et al.*, 2002). Virulent isolates of *P. parasitica* produce low to negligible amounts of elicitins in tobacco, thereby evading pathogen-recognition mechanisms of the host (Colas *et al.*, 2001).

Elicitins, when applied to the vasculature, can also induce SAR (Picard *et al.*, 2000; Baillieul *et al.*, 2003; Cordelier *et al.*, 2003). In concurrence with their ability to activate SAR, ectopic expression of elicitins in transgenic tobacco conferred resistance to a variety of pathogens (Keller *et al.*, 1999). Studies with radiolabeled elicitin demonstrated that these can move from the inoculation site on a stem towards leaves (Zanetti *et al.*, 1992). Together, these results suggest that elicitins, or a factor produced in plants exposed to elicitins, can move systemically through plants. Plant LTP1 and elicitins compete for a cell surface receptor in tobacco (Buhot *et al.*, 2001), suggesting that plant LTPs may also have a role in defense signaling. Indeed, in *Arabidopsis*, the activation of SAR requires the DIR1 (DEFECTIVE IN INDUCED RESISTANCE1) protein which is an LTP (Maldonado *et al.*, 2002). Petiole exudates collected from leaves of avirulent pathogen-inoculated *dir1* plants were unable to

activate SAR when applied to wild-type plants. In contrast, the *dir1* mutant was responsive to the SAR signal present in petiole exudates of avirulent pathogen-challenged wild-type leaves, suggesting that the *dir1* mutant is unable to accumulate a long-distance signal involved in activation of SAR. The SAR deficiency of the *Arabidopsis* lipid-biosynthesis mutants, *sfd1* and *fad7*, is also due to their inability to accumulate an SAR-promoting factor in petiole exudates (Chaturvedi *et al.*, 2008). However, the SAR-promoting activity was reconstituted by mixing petiole exudates from avirulent pathogen-inoculated *dir1* leaves with similarly derived exudates from the *sfd1* and *fad7* mutants, indicating that DIR1 plus a SFD1/FAD7-dependent factor are required together in the petiole exudates for long-distance SAR signaling (Chaturvedi *et al.*, 2008). Indeed, DIR1 was recently shown to contain a lipid-binding pocket that is capable of binding lipids (Lascombe *et al.*, 2006). Whether an SFD1/FAD7-derived factor binds DIR1 in the petiole exudates needs to be tested.

10.8 Concluding remarks

During the last 10 years significant strides have been made in identifying new signaling lipids, elucidating routes of biosynthesis and uncovering novel functions for previously described lipids. However, barring a few lipids, the physiological roles of many lipids in plant–pathogen interaction remain to be elucidated. Some of these lipids and associated processes could provide important targets for controlling plant disease.

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Chapter 11

INDUCED RESISTANCE – ORCHESTRATING DEFENCE MECHANISMS THROUGH CROSSTALK AND PRIMING

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Abstract: In nature, plants interact with a wide range of microbial pathogens and herbivorous insects. During the evolutionary arms race between plants and their attackers, primary and secondary immune responses evolved to recognise common or highly specialised features of the attacker encountered, resulting in sophisticated mechanisms of induced defence. Induced resistance mechanisms are characterised by a broad-spectrum effectiveness and often act systemically in plant parts distant from the site of primary attack, thereby protecting the plant against subsequent invaders. Plant hormones are key players in the regulation of the defence signalling pathways involved. Because induced defence responses entail ecological fitness costs, plants must possess elaborate regulatory mechanisms that efficiently coordinate the activation of attacker-specific defences so that fitness costs are minimised while optimal resistance is attained. A major focus in plant defence signalling research is to uncover key mechanisms by which plants tailor their responses to different attackers, and to investigate how plants cope with simultaneous interactions with multiple aggressors. Pathway crosstalk and priming for enhanced defence emerged as important regulatory mechanisms that enhance the efficiency of the plant's inducible defence arsenal. Here, we review the current knowledge on the signalling cascades involved in different types of induced pathogen and insect resistance, and the regulatory mechanisms by which plants are able to orchestrate their inducible defences in a cost-effective manner.

Keywords: crosstalk; priming; induced resistance; defence signalling; phytohormones

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11.1 Introduction

11.1.1 Constitutive defence

Plants are abundant on earth and form the basis of most food webs. Each of the ~300 000 plant species interacts with a range of organisms, some of which are harmful (e.g. pathogens or herbivorous insects) and others beneficial (e.g. growth-promoting rhizobacteria, mycorrhizal fungi and predatory enemies of herbivores). Intriguingly, plants are resistant to the majority of their attackers. This resistance is based on an array of defensive mechanisms. Some of these mechanisms are pre-existing and prevent or attenuate the invasion of potential attackers. Thorns, needles and trichomes are examples of constitutively present defensive structures that are designed to harm or deter herbivores. On a smaller scale, the cell wall poses a pre-existing physical barrier to many microorganisms. In addition, plants constitutively produce secondary metabolites that inhibit growth of microbes or render the tissue less attractive for herbivores (Osbourn, 1996; Tierens *et al.*, 2001).

11.1.2 PAMP-triggered immunity

Despite the diversity of pre-existing defensive barriers, many microbes and insects succeed in breaking through this first layer of defence. However, as a second line of defence, a wide spectrum of inducible plant defences becomes activated that help the plant to prevent the attacker from causing further damage, either by blocking its colonisation or by directly targeting the attacker's physiology (see Chapter 8). For this second line of defence, plants have evolved sophisticated strategies to 'perceive' their attacker and translate this 'perception' into an effective defence response (Chisholm *et al.*, 2006; Jones and Dangl, 2006). This primary immune response recognises common features of microbial pathogens, such as flagellin, chitin, glycoproteins and lipopolysaccharides (Bittel and Robatzek, 2007). These microbial determinants are referred to as pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) (Chisholm *et al.*, 2006; Jones and Dangl, 2006; Bittel and Robatzek, 2007). PAMPs activate pattern-recognition receptors, which in turn initiate diverse downstream signalling events that ultimately result in the activation of a basal resistance that is called PAMP-triggered immunity (PTI; Chisholm *et al.*, 2006; Jones and Dangl, 2006) (see Chapter 2).

11.1.3 Effector-triggered immunity

During the co-evolutionary arms race between plants and their microbial attackers, pathogens acquired the ability to suppress PTI via the delivery of specific effector proteins. In turn, plants acquired immune receptors (R proteins) that are able to recognise these attacker-specific effector proteins

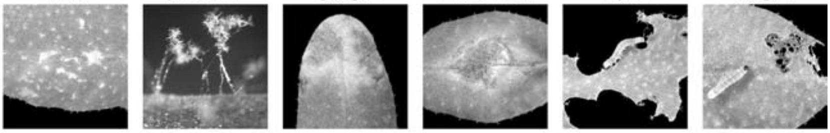
(in this context known as avirulence proteins), resulting in a second line of defence called 'effector-triggered immunity' (ETI; Chisholm *et al.*, 2006; Jones and Dangl, 2006). ETI results in *R* gene-mediated resistance which is often associated with a highly effective hypersensitive response (HR) that arrests further pathogen ingress. The co-evolutionary arms race between plants and herbivorous insects has been intensely debated (Schoonhoven *et al.*, 2005). However, knowledge of the underlying molecular mechanisms is relatively limited in comparison to well-studied mechanisms involved in the arms race between pathogens and their host plants.

11.1.4 Systemically induced resistance

In addition to PTI and ETI that act locally and are activated upon attacker-specific recognition, plants can activate yet another line of defence that is referred to as 'induced resistance'. Over the past three decades, distinct forms of induced resistance have been identified, mainly defined by differences in the signal transduction pathways. Induced resistance can be activated by microbial pathogens and insect herbivores, but also by beneficial microorganisms, such as mycorrhizal fungi and plant growth-promoting rhizobacteria (Kessler and Baldwin, 2002; Dicke and Hilker, 2003; Pozo *et al.*, 2004). While PTI and ETI are thought to be directed specifically against the microbial invader encountered, induced resistance is typically characterised by a broad spectrum of effectiveness. Moreover, induced resistance often also acts systemically in plant parts distant from the site of primary attack, thereby protecting the plant against subsequent invaders. Systemically induced resistance can be activated upon a local PTI or ETI response, but there are also other means of activating systemic-induced resistance. Several biologically induced systemic defence responses have been characterised in detail: systemic acquired resistance (SAR), which is triggered by pathogens causing limited infection, such as hypersensitive necrosis (Durrant and Dong, 2004); rhizobacteria-induced systemic resistance (ISR), which is activated upon colonisation of roots by selected strains of non-pathogenic rhizobacteria (Van Loon *et al.*, 1998), and wound-induced resistance (WIR), which is typically elicited upon tissue damage, such as caused by insect feeding (Kessler and Baldwin, 2002; Howe, 2004). In addition, broad-spectrum resistance can be induced by chemicals such as the non-protein amino acid β-aminobutyric acid (BABA) (Zimmerli *et al.*, 2000). An overview of the spectrum of effectiveness of four types of induced resistance in *Arabidopsis* is presented in Fig. 11.1.

11.1.5 Hormonal regulation of induced resistance

The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) emerged as key players in the regulation of signalling pathways involved in these induced defence responses (Durrant and Dong, 2004; Van Poecke and Dicke, 2004; Von Dahl and Baldwin, 2007). Other plant hormones,



	Turnip crinkle virus	*Hyaloperonospora parasitica*	*Pseudomonas syringae*	*Alternaria brassicicola*	*Pieris rapae*	*Spodoptera exigua*
SAR	+	+	+	–	–	+
ISR	–	+/–	+	+	–	+
WIR	+	nd	+	–	+	nd
BABA-IR	nd	+	+	+	nd	nd

Figure 11.1 Spectrum of effectiveness of systemically induced resistance in *Arabidopsis thaliana*. Induced resistance is indicated by + and ineffective resistance by – (nd; not determined). SAR is effective against (hemi-)biotrophic pathogens such as turnip crinkle virus (TCV), *Hyaloperonospora parasitica* and *Pseudomonas syringae* (Ton *et al.*, 2002b). By contrast, ISR is not effective against TCV, but is effective against necrotrophic pathogens such as *Alternaria brassicicola* (Ton *et al.*, 2002b). Both SAR and ISR are effective against the generalist herbivore *Spodoptera exigua*, whereas the specialist herbivore *Pieris rapae* is unaffected by both induced resistance responses (Van Oosten, 2008). WIR, induced by *P. rapae* caterpillars, confers resistance against subsequent infestation by *P. rapae* and against TCV and *P. syringae*, but not against *A. brassicicola* (De Vos *et al.*, 2006). BABA-IR is effective against all attackers tested (Zimmerli *et al.*, 2000; Ton and Mauch-Mani, 2004). BABA-IR, β-aminobutyric acid-induced resistance; ISR, induced systemic resistance; SAR, systemic acquired resistance; WIR, wound-induced resistance.

including abscisic acid (ABA) (Mauch-Mani and Mauch, 2005), brassinosteroids (Nakashita *et al.*, 2003) and auxins (Navarro *et al.*, 2006; Wang *et al.*, 2007), have also been reported to have a role in induced plant defence responses, but their significance is less well studied. Upon pathogen or insect attack, plants respond with the production of a specific blend of these alarm signals which varies greatly in quantity, composition and timing, and results in the activation of differential sets of defence-related genes that eventually determine the nature of the defence response against the attacker encountered (Reymond and Farmer, 1998; Rojo *et al.*, 2003; De Vos *et al.*, 2005). Global expression profiling of various *Arabidopsis*-attacker interactions revealed substantial crosstalk between SA-, JA- and ET-dependent defence pathways (Glazebrook *et al.*, 2003; De Vos *et al.*, 2005). Cross-communication between these pathways provides a powerful regulatory potential that allows the plant to fine-tune its defence responses.

11.1.6 Priming for enhanced defence

Induced resistance is often associated with the production of defensive compounds such as pathogenesis-related (PR) proteins with anti-microbial activity (Van Loon *et al.*, 2006), proteinase inhibitors (PIs) that affect insect feeding (Howe, 2004) or volatiles that attract parasitoids and predators of the

herbivores that feed on the plant (Van Poecke and Dicke, 2004). However, in many cases, the enhanced defensive capacity in induced plants cannot be attributed to direct activation of defence-related genes. Instead, broad-spectrum protection of an induced plant is based on a faster and stronger activation of basal defence mechanisms upon exposure to either microbial pathogens or herbivorous insects. It is therefore hypothesised that the broad-spectrum characteristic of induced resistance is largely based on this conditioning of the tissue to react more effectively to a stress condition. By analogy with a phenotypically similar phenomenon in animals and humans, this enhanced capacity to express basal defence mechanisms is called 'priming' (Conrath *et al.*, 2002, 2006).

In this chapter, we aim to review the current status of induced disease resistance signalling research. We focus on the roles of SA, JA and ET in the signalling cascades involved in the different types of induced resistance. We also discuss two regulatory mechanisms that enhance the efficiency of the plant's inducible defence arsenal: cross-communication between defence signalling pathways and priming of induced defence.

11.2 Induced resistance signalling

11.2.1 Systemic acquired resistance

The classical form of induced resistance is called systemic acquired resistance (SAR) and develops in systemic tissue upon primary infection with a necrosis-inducing pathogen (Durrant and Dong, 2004). Avirulent pathogens that activate ETI resulting in an HR are potent inducers of SAR. However, PTI activated by PAMPs that induce the SA signalling pathway can also trigger SAR (Mishina and Zeier, 2006). SAR has been demonstrated in many plant–pathogen interactions (Ryals *et al.*, 1996; Sticher *et al.*, 1997) and is typically characterised by a restriction of pathogen growth and suppression of disease symptom development compared to non-induced plants infected by the same pathogen (Hammerschmidt, 1999). Although SAR is effective against a broad range of pathogens, including viruses, bacteria, fungi and oomycetes (Kuc, 1982), it seems predominantly effective against pathogens with a (hemi-)biotrophic lifestyle (Ton *et al.*, 2002b).

11.2.1.1 The onset of SAR

The onset of SAR is accompanied by a local and systemic increase in endogenous levels of SA (Malamy *et al.*, 1990; Métraux *et al.*, 1990). Moreover, SAR is associated with the coordinate activation of a specific set of genes encoding PR proteins, some of which possess antimicrobial activity (Van Loon *et al.*, 2006). Exogenous application of SA or its functional analogues 2,6-dichloroisonicotinic acid (INA) or benzothiadiazole (BTH) induces SAR and

activates the same set of *PR* genes (Ryals *et al.*, 1996). The importance of SA in the regulation of SAR became evident through experiments with transgenic NahG plants that convert SA into catechol through the activity of an introduced bacterial salicylate hydroxylase (*NahG*) gene. Transgenic NahG plants that cannot accumulate SA are incapable of developing SAR and do not show *PR* gene activation upon pathogen infection (Gaffney *et al.*, 1993; Lawton *et al.*, 1995), indicating that SA is a necessary intermediate in the SAR signalling pathway. Interestingly, NahG tobacco and *Arabidopsis* plants are not only incapable of accumulating SA and mounting SAR but also more susceptible to many different pathogens, including bacteria, viruses, fungi and oomycetes (Delaney *et al.*, 1994; Kachroo *et al.*, 2000). Similarly, *Arabidopsis* mutants that are not able to enhance the production of SA upon pathogen infection, such as *eds1* (*enhanced disease susceptibility 1*), *sid1* (*salicylic acid induction-deficient 1*) (*eds5*), *sid2* (*eds16*) and *pad4* (*phytoalexin-deficient 4*), display a higher level of susceptibility to different pathogens, indicating that SA also plays an important role in basal defence (Rogers and Ausubel, 1997; Zhou *et al.*, 1998; Nawrath and Métraux, 1999; Feys *et al.*, 2001; Wildermuth *et al.*, 2001).

Although SA is an essential molecule in the signal transduction of SAR, it seems that this molecule does not function as the systemically transported signal of SAR. Using grafts of wild-type and SA-degrading NahG tobacco rootstocks and scions, Vernooij *et al.* (1994) demonstrated that SA production is not required for the generation of the mobile signal in SAR. On the other hand, Shulaev *et al.* (1995) demonstrated that radioactively labelled SA, synthesised at the site of primary infection, is transported throughout the plant. Seskar *et al.* (1998) proposed that methyl salicylate (MeSA), synthesised from SA in the locally infected leaves and systemically transported throughout the plant, acts in the systemic target tissues by being converted back to SA. This hypothesis was supported by later findings in tobacco, demonstrating that SA methyl transferase and the MeSA esterase SA-BINDING PROTEIN 2 are essential for the expression of SAR in locally infected and systemic leaves, respectively (Kumar and Klessig, 2003; Forouhar *et al.*, 2005; Park *et al.*, 2007).

11.2.1.2 Lipid-derived components involved in SAR

Besides SA and MeSA, lipid-derived components have been implicated as important systemic signals during SAR. Analysis of an *Arabidopsis* T-DNA insertion line identified the *DEFECTIVE IN INDUCED RESISTANCE 1* (*DIR1*) gene encoding a putative apoplastic lipid transfer protein required for pathogen-induced SAR (Maldonado *et al.*, 2002). Assessment of the ability of petiole exudates from wild-type and mutant *dir1* plants to induce SAR gene expression indicated that *dir1* mutant plants are incapable of producing or transmitting the mobile signal that is essential for the systemic expression of SAR. Remarkably, *dir1* still accumulated SA during the establishment and manifestation stages of SAR. The authors suggested that DIR1 interacts with a lipid-derived molecule to allow long-distance signalling. Hence, analogous

to the agonist function of SA in ETI (Shirasu *et al.*, 1997), the role of SA in SAR might be to amplify a DIR1-dependent signal. Moreover, SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY 1 (SFD1), required for systemic activation of SAR, also appeared to be involved in lipid metabolism (Nandi *et al.*, 2004). Furthermore, Truman *et al.* (2007) suggested a central role for jasmonates in SAR signalling. However, other lines of evidence demonstrate that mutants disrupted in JA signalling, e.g. *jar1* (*jasmonate resistant 1*) and *eds8*, are still able to mount wild-type levels of SAR (Pieterse *et al.*, 1998; Ton *et al.*, 2002a). Therefore, the exact role for JAs in SAR needs to be explored further.

11.2.1.3 SAR signal transduction

Transduction of the SA signal to activate *PR* gene expression and SAR requires the function of the regulatory protein NPR1 (NONEXPRESSOR OF *PR*-GENES 1), also known as NIM1 (NON IMMUNITY 1), or SAI1 (SALICYLIC ACID-INSENSITIVE 1) (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). Mutations in the *NPR1* gene render the plant largely unresponsive to pathogen-induced SA production, thereby blocking the induction of SA-dependent *PR* genes and SAR (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). *NPR1* is expressed throughout the plant at low levels and shows only modest induction upon pathogen infection or SA treatment in wild-type *Arabidopsis* (Cao *et al.*, 1997; Ryals *et al.*, 1997). Overexpression of NPR1 does not result in a massive induction of the *PR-1* gene, indicating that NPR1 requires post-translational activation in order to transduce the SA signal (Cao *et al.*, 1998; Friedrich *et al.*, 2001). Indeed, SA-induced redox changes have been shown to reduce intermolecular disulphide bonds that hold NPR1 together as an inactive oligomer. This reduction converts the inactive oligomeric complex into an active monomeric form which is translocated into the nucleus to activate *PR* gene expression (Mou *et al.*, 2003).

Although NPR1 acts as a modulator of *PR* gene expression, it does not bind to DNA itself (Cao *et al.*, 1997). Yeast two-hybrid analyses indicated that NPR1 functions through members of the TGA subclass of the basic leucine zipper (bZIP) family of transcription factors (TGAs) that bind to the *as-1* promoter element of the *PR-1* gene (Dong, 2004). Electromobility shift assays showed that NPR1 substantially increases binding of TGA2 to SA-responsive promoter elements in the *Arabidopsis PR-1* gene (Després *et al.*, 2000), suggesting that NPR1-mediated DNA binding of TGAs is important for *PR* gene activation. TGA factors act either as positive or as negative regulators of *PR-1* gene expression (Kesarwani *et al.*, 2007), indicating that the *PR* gene expression during SAR must be under tight regulatory control. Recently, a genomics-directed approach demonstrated that upon induction of SAR, a select group of *WRKY* transcription factor genes is induced after nuclear translocation of NPR1 monomers (Wang *et al.*, 2006). Like the TGAs, WRKY transcription factors have also either a positive or a negative effect on the expression of *PR* genes, thereby further contributing to the complexity

of the SA- and NPR1-dependent signalling network involved in SAR (Wang *et al.*, 2006).

In addition to the regulation of *PR* genes, NPR1 was shown to target the transcription of genes that are involved in the protein secretory pathway. Expression of these proteins ensures proper processing of *PR* transcripts and secretion of PR proteins which contributes to SA-based resistance (Wang *et al.*, 2005). Other important regulators involved in NPR1-dependent *PR* gene expression and SAR have been identified (e.g. NIMIN1, SNI1). Their role in defence signalling will be discussed elsewhere in this book.

Recently, a novel signalling component in SAR was identified. Mishina and Zeier (2006) demonstrated in *Arabidopsis* that FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) plays a critical role in the onset of SAR. Transcription of the *FMO1* gene was induced locally and systemically upon inoculation with an avirulent strain of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Analysis of a T-DNA knockout mutant revealed that FMO1 is important in the amplification of the SAR signal in the systemic tissues (Mishina and Zeier, 2006). A future challenge represents further characterisation of the FMO1-dependent pathway, and how this pathway exerts its role in the onset of the systemically expressed resistance during SAR.

Besides being important for systemic SAR signalling (Koch *et al.*, 2006), FMO1 contributes local basal resistance against *Hyaloperonospora parasitica* and *P. syringae* (Bartsch *et al.*, 2006). Bartsch *et al.* (2006) demonstrated that FMO1 mediates an SA-independent branch of EDS1 signalling. In some studies, *eds1* mutant plants were also found to be defective in SAR signalling (Truman *et al.*, 2007; C. Vlot, L. Jorda and J.E. Parker, unpublished results), while in another report (Mishina and Zeier, 2006) EDS1 appeared not to contribute to SAR. Hence, the roles of EDS1 and FMO1 in SAR need further clarification.

11.2.2 Induced systemic resistance

Beneficial microorganisms, such as mycorrhizal fungi and non-pathogenic rhizobacteria, have been demonstrated to induce a systemic resistance response that is phenotypically similar to SAR (Van Loon *et al.*, 1998; Pozo *et al.*, 2004). Large numbers of non-pathogenic rhizobacteria are attracted to plant exudates produced at the surface of plant roots. Selected strains of these non-pathogenic rhizobacteria are capable of stimulating plant growth (Bloemberg and Lugtenberg, 2001). Growth promotion results mainly from the suppression of soilborne pathogens and other deleterious microorganisms. Fluorescent *Pseudomonas* spp. are among the most effective plant growth-promoting rhizobacteria and have been shown to be responsible for the reduction of soilborne diseases in naturally disease-suppressive soils (Weller *et al.*, 2002). This type of natural biological control can result from competition for nutrients, siderophore-mediated competition for iron, production of antibiotic compounds or production of lytic enzymes (Van Loon and Bakker, 2005).

Apart from such direct antagonistic effects on soilborne pathogens, some rhizobacterial strains are capable of reducing disease incidence in above-ground plant parts through a plant-mediated defence mechanism called ISR (Van Loon *et al.*, 1998). Like SAR, rhizobacteria-mediated ISR has been demonstrated in many plant species, e.g. bean, carnation, cucumber, radish, tobacco, tomato and *Arabidopsis*, and is effective against a broad spectrum of plant pathogens, including oomycetes, fungi, bacteria and viruses (Van Loon *et al.*, 1998). While SAR is predominantly operative against biotrophic pathogens that are resisted through SA-dependent defences, ISR also functions against necrotrophic pathogens that are susceptible to JA-dependent responses, such as *Alternaria brassicicola* (Ton *et al.*, 2002b).

11.2.2.1 The onset of ISR

In contrast to SAR, relatively little is known about the onset of ISR. Although ISR-inducing rhizobacteria show little specificity in their colonisation of roots of different plant species (Van Loon *et al.*, 1998), their ability to induce ISR is dependent on the bacterium–host combination. For instance, *Pseudomonas fluorescens* WCS374r is capable of inducing ISR in radish but not in *Arabidopsis* (Leeman *et al.*, 1995; Van Wees *et al.*, 1997). Conversely, *Arabidopsis* is responsive to *Pseudomonas putida* WCS358r, whereas radish is not (Leeman *et al.*, 1995; Van Wees *et al.*, 1997). *P. fluorescens* WCS417r is capable of inducing ISR in both *Arabidopsis* and radish (Van Wees *et al.*, 1997) as well as in other species, e.g. carnation, radish, tomato and bean but not in *Eucalyptus* (Van Loon and Bakker, 2005). Besides interspecies differences in ISR-inducibility, intraspecies variation is also observed. *Arabidopsis* accessions Columbia (Col-0) and Landsberg *erecta* (L*er*-0) are both responsive to ISR induction by WCS417r but accessions Wassilewskija (Ws-0) and RLD1 are not (Van Wees *et al.*, 1997; Ton *et al.*, 1999). These latter accessions are compromised in a common trait governing a step between the recognition of the bacterium and the expression of ISR. These data clearly indicate that ISR is genetically determined.

Several bacterially derived MAMPs have been implicated in the elicitation of rhizobacteria-mediated ISR (Van Loon and Bakker, 2005). Examples are flagella, cell wall components such as lipopolysaccharides and excreted metabolites such as siderophores and antibiotics. Although conclusive evidence is still lacking, the striking homologies with sensitive perception mechanisms for pathogen-derived PAMPs that function in PTI suggest that non-pathogenic rhizobacteria are similarly recognised by general immune surveillance mechanisms.

Research on the molecular mechanism of rhizobacteria-mediated ISR was initially focused on the role of PR proteins, as the accumulation of these proteins was considered to be strictly correlated with induced disease resistance. However, radish plants whose roots were treated with ISR-inducing WCS417r bacteria did not accumulate PR proteins, although these plants clearly showed enhanced resistance against *Fusarium* wilt disease (Hoffland

et al., 1995). Similarly, *Arabidopsis* plants expressing WCS417r-mediated ISR showed enhanced resistance against *Fusarium oxysporum* f.sp. *raphani* and *Pst* DC3000, but this did not coincide with the activation of the SAR marker genes *PR-1*, *PR-2* and *PR-5* (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Determination of SA levels in ISR-expressing *Arabidopsis* plants revealed that ISR is not associated with increased accumulation of SA (Pieterse *et al.*, 2000). Moreover, WCS417r-mediated ISR was expressed normally in SA non-accumulating *Arabidopsis* NahG plants (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). This led to the conclusion that *P. fluorescens* WCS417r-mediated ISR is an SA-independent resistance mechanism and that WCS417r-mediated ISR and pathogen-induced SAR are regulated by distinct signalling pathways. SA-independent ISR has been shown not only in *Arabidopsis* (Pieterse *et al.*, 1996; Iavicoli *et al.*, 2003; Ryu *et al.*, 2003) but also in tobacco (Press *et al.*, 1997; Zhang *et al.*, 2002) and tomato (Yan *et al.*, 2002). The wide range of induction of ISR suggests that the ability of these *Pseudomonas* strains to activate an SA-independent pathway controlling systemic resistance is a common feature of these non-pathogenic rhizobacteria.

11.2.2.2 ISR signal transduction

Since SA is not required for WCS417r-elicited ISR, the *Arabidopsis* JA response mutant *jar1* and the ET response mutant *etr1* (*ethylene response 1*) were tested for their ability to express ISR. Both mutants were unable to mount resistance against *Pst* DC3000 after colonisation of the roots by WCS417r (Pieterse *et al.*, 1998), indicating that ISR requires responsiveness to both JA and ET. Another indication for involvement of the JA signalling pathway came from the analysis of *Arabidopsis* mutant *eds8* which was previously shown to exhibit enhanced susceptibility to *P. syringae* (Glazebrook *et al.*, 1996). This mutant was impaired in both WCS417r-mediated ISR (Ton *et al.*, 2002a) and JA signalling (Ton *et al.*, 2002b; Glazebrook *et al.*, 2003). To elucidate further the role of ET in the ISR signalling pathway, a large set of well-characterised ET signalling mutants was analysed. None of these mutants showed an ISR response against *Pst* DC3000 after colonisation of the roots by WCS417r (Knoester *et al.*, 1999). These results confirmed that an intact ET signalling pathway is required for the establishment of ISR. Particularly interesting was the analysis of the *eir1* (*ethylene insensitive root 1*) mutant, which is ET insensitive in the roots, but not in the shoot. This *eir1* mutant was incapable of showing ISR after root colonisation by WCS417r. In contrast, after leaf infiltration with WCS417r it exhibited ISR, indicating that responsiveness to ET is required at the site of rhizobacterial induction (Knoester *et al.*, 1999).

To investigate possible involvement of the SAR regulatory protein NPR1 in ISR signalling, the *Arabidopsis npr1* mutant was tested in the ISR bioassay. Surprisingly, *npr1* was incapable of displaying WCS417r-mediated ISR (Pieterse *et al.*, 1998; Van Wees *et al.*, 2000). This result showed that WCS417r-mediated ISR, like SA-dependent SAR, is an NPR1-dependent defence response. Further analysis of the ISR signal transduction pathway revealed that NPR1

acts downstream of the JA- and ET-dependent steps (Pieterse *et al.*, 1998). Because SAR is associated with NPR1-dependent *PR* gene expression, and ISR is not, the action of NPR1 in ISR must be different from that in SAR. These different activities are not mutually exclusive because simultaneous activation of ISR and SAR can lead to an enhanced defensive activity compared to that observed with either type of induced resistance alone (Van Wees *et al.*, 2000). These results suggest that the NPR1 protein is important in regulating and connecting different hormone-dependent defence pathways. As ISR is neither dependent on SA nor associated with *PR* gene expression, the function of NPR1 in ISR is still unknown. Recently, Wang *et al.* (2005) demonstrated a role for NPR1 in the stress-induced augmentation of the protein secretory pathway. Since NPR1 influences the transcription rate of genes involved in protein secretion differently from that of *PR* genes (Wang *et al.*, 2005), it is tempting to speculate that NPR1-dependent stimulation of this process may also be involved in the expression of rhizobacteria-mediated ISR.

Microarray analysis revealed that the R2R3-MYB-like transcription factor gene *MYB72* is specifically activated in roots of *Arabidopsis* upon colonisation by WCS417r (Verhagen *et al.*, 2004). T-DNA knockout mutants *myb72-1* and *myb72-2* appeared to be incapable of mounting ISR against different pathogens, indicating that MYB72 is an essential ISR signalling component that is required in the roots during early signalling steps of broad-spectrum ISR (Van der Ent *et al.*, 2008).

Although the majority of ISR-inducing rhizobacterial strains tested to date trigger an SA-independent signalling pathway, some exceptions have been reported (Van Loon and Bakker, 2005). For instance, an SA-overproducing mutant of *Pseudomonas aeruginosa* 7NSK2 was shown to trigger the SA-dependent SAR pathway in bean and tobacco by producing SA at the root surface (De Meyer *et al.*, 1999). In *Arabidopsis*, the rhizobacterial strain *Paenibacillus alvei* K165 induced systemic resistance against *Verticillium dahliae* which was blocked in SA signalling mutants *eds5* and *sid2* (Tjamos *et al.*, 2005), indicating that this rhizobacterial strain activated an SA-dependent defence pathway.

11.2.3 Induced resistance against herbivorous insects

To fend off insect herbivores, plants have adapted two distinct strategies: induced defence directed against the attacker, referred to as direct defence, and induced defence aimed at exploiting the natural enemies of the attacker, referred to as indirect defence. Both types of defence can be triggered upon insect feeding. Direct defence includes induced responses such as the production of secondary chemicals or enzymes that act as toxins or feeding deterrents (Kessler and Baldwin, 2002; Howe, 2004), whereas indirect defence can involve production of a blend of volatiles that attracts predatory or parasitic enemies of the herbivores (Turlings and Ton, 2006).

11.2.3.1 Direct defence

One of the best-studied examples of induced direct defence against herbivores is the rapid and systemic induction of PIs after wounding or insect feeding in tomato (*Lycopersicon esculentum*) (Howe, 2004). Upon consumption of induced tissues by the herbivore, PIs bind to and inhibit digestive proteases in the insect gut, leading to reduced feeding (Farmer and Ryan, 1992). Several PI-inducing signals have been discovered including oligogalacturonides (OGAs) and systemin. In response to wounding, OGAs are produced from cell wall components, and the 18-amino acid peptide systemin is generated by cleavage from its precursor protein prosystemin. This eventually leads to JA synthesis via the octadecanoid pathway and induction of PIs and other defence genes (Farmer and Ryan, 1992). However, the signal transduction events that couple perception of OGAs and systemin at the plasma membrane to the subsequent activation of JA synthesis in the chloroplast remain to be elucidated (Howe, 2004).

The key role of JAs in induced direct defence against insect herbivores has been demonstrated in many plant–herbivore interactions. For instance, *Pieris rapae* caterpillars (small cabbage white butterfly) gained significantly more weight when they fed on the *Arabidopsis* JA signalling mutant *coi1* (*coronatine insensitive 1*) than on wild-type plants (Reymond *et al.*, 2004). Likewise, the population of the aphid *Myzus persicae* (green peach aphid) increased faster on *coi1* than on wild-type *Arabidopsis* (Ellis *et al.*, 2002). Conversely, on the constitutive JA signalling *Arabidopsis* mutant *cev1* (*constitutive expression of VSP1*), population levels of *M. persicae* were reduced (Ellis *et al.*, 2002). The tomato mutant *def1* (*defenseless 1*), deficient in JA biosynthesis, has compromised resistance to tissue-chewing *Manduca sexta* (tobacco hornworm) and *Spodoptera exigua* (beet armyworm) larvae as well as to the cell-content feeding *Tetranychus urticae* (two-spotted spider mite) and *Frankliniella occidentalis* (Western flower thrips) (Howe *et al.*, 1996; Li *et al.*, 2002; Thaler *et al.*, 2002). The JA biosynthesis mutant *fad3fad7fad8* (*fatty acid desaturation 3, 7, 8*) of *Arabidopsis* is extremely sensitive to larvae of the fungal gnat, *Bradysia impatiens* (McConn *et al.*, 1997). Moreover, the ET insensitive *Arabidopsis* mutant *ein2* is less resistant to larvae of *Spodoptera littoralis* (Egyptian cotton worm) (Stotz *et al.*, 2000). In addition, *Arabidopsis* mutants and transgenics that are compromised in SA-dependent defence responses exhibit enhanced resistance against feeding by the cabbage looper *Trichoplusia ni* (Cui *et al.*, 2002). Thus, whereas JA plays a main role, ET and SA also influence plant resistance against insects.

11.2.3.2 Indirect defence

Insect feeding induces production of volatile chemicals in the plant which are effective in attracting parasitic and predatory insects (Van Poecke and Dicke, 2004; Turlings and Ton, 2006). JA is the major signalling molecule involved in the induced production of plant volatiles (Van Poecke and Dicke *et al.*, 2004). Treatment of plants with JA leads to the emission of a volatile blend that is similar, but not identical, to the blend of herbivore-infested plants. Moreover,

the volatiles induced by JA treatment are attractive to carnivorous enemies of the herbivores (Van Poecke and Dicke, 2004).

ET and SA can also play a role in indirect defence. ET was shown to enhance JA-mediated volatile emission in lima bean (*Phaseolus lunatus*) (Horiuchi *et al.*, 2001). Herbivores such as spider mites induce the emission of MeSA in many plant species (Ament *et al.*, 2004; De Boer and Dicke, 2004), which can lead to the activation of SA-inducible defence-related genes (Arimura *et al.*, 2000; Kant *et al.*, 2004). In line with these results, feeding by *P. rapae* larvae induced MeSA production in *Arabidopsis* (Van Poecke and Dicke, 2002). In *Arabidopsis* NahG plants, MeSA was not produced upon *P. rapae* feeding, leading to decreased attractiveness of the induced volatile blend to the parasitoid wasp *Cotesia rubecula* (Van Poecke and Dicke, 2002). Similarly, the volatiles induced upon feeding of *P. rapae* in the transgenic *Arabidopsis* S-12 line with reduced JA biosynthesis were less attractive to *C. rubecula* (Van Poecke and Dicke, 2002). These results illustrate that JA, ET and SA all play a role in induced indirect defence against insects.

Airborne volatile organic compounds (VOCs) that are produced upon insect herbivory not only are important in the attraction of natural enemies of the herbivore but can also enhance the level of resistance in neighbouring plants that is effective against future insect attack (Baldwin *et al.*, 2006; Turlings and Ton, 2006). From an evolutionary perspective, it has always been puzzling how this form of plant–plant communication can persist, as it benefits the receiver plant, rather than the emitter plant. It seems more likely therefore that VOCs play an important role as systemic within-plant signalling components in insect-induced resistance. Indeed, recent findings by Heil and Silva Bueno (2007) and Frost *et al.* (2007) support a within-plant signalling role of VOCs. Surrounding organisms, such as neighbouring plants, herbivorous insects and predatory or parasitoid insects, have merely evolved the ability to 'eavesdrop' on this airborne within-plant signalling.

11.2.3.3 JA signal transduction

In the past 20 years, JA and its functionally active derivates (e.g. JA isoleucine (JA-Ile) and methyl JA (MeJA)) emerged as important regulators of induced plant defence. JAs are produced by the octadecanoid pathway after insect herbivory or pathogen attack (Wasternack, 2007). Downstream target genes include defence-related genes such as the defensin *PDF1.2* (*PLANT DEFENSIN 1.2*) and thionin *Thi2.1* (*THIONIN 2.1*), but also genes that are required for the biosynthesis of JA. All plant responses to JA that have been described so far are dependent on an intact COI1 protein (Feys *et al.*, 1994; Xie *et al.*, 1998). COI1 encodes an F-box protein (Xie *et al.*, 1998) which is part of an SCF^{COI1} E3 ubiquitin ligase complex that is involved in proteasome-mediated protein degradation (Xu *et al.*, 2002). The F-box protein confers specificity to the E3 ligase complex by interacting with proteins that are targeted for ubiquitination and subsequent degradation. Therefore, COI1 is thought to mediate the removal

of repressors that keep JA responses inactive (Devoto *et al.*, 2003). Recently, JAZ (jasmonate ZIM-domain) proteins have been identified as likely candidates for COI1-targeted transcriptional repressors of JA-responsive genes (Chini *et al.*, 2007; Thines *et al.*, 2007). JAZ proteins repress JA-responsive gene expression by active suppression of transcriptional activators of JA-responsive genes such as AtMYC2 (Chini *et al.*, 2007). Upon stimulation of the JA response, the physical interaction of JA-Ile with JAZ proteins allows COI1 to target JAZ proteins for degradation by the proteasome (Thines *et al.*, 2007). As a result, repression by the JAZ proteins is lifted, causing enhanced transcription of JA-responsive genes. Notably, JAZ biosynthesis genes are induced by JA themselves, indicating a negative feedback loop that allows for a pulsed response to the JA-inducing stimulus (Chini *et al.*, 2007; Thines *et al.*, 2007).

11.2.4 Chemically induced resistance

In addition to biological stimuli, the application of certain chemicals can induce resistance of distal plants parts. Often, these chemicals induce a similar resistance response as biologically induced SAR, as is the case with applications of INA, BTH and SA (Durrant and Dong, 2004). However, the non-protein amino acid BABA seems to induce a somewhat different induced resistance response. Application of BABA induces resistance in many different plant species (Jakab *et al.*, 2001; Cohen, 2002) and is effective against biotrophic and necrotrophic pathogens (Siegrist *et al.*, 2000; Zimmerli *et al.*, 2000; Ton and Mauch-Mani, 2004), insects (Hodge *et al.*, 2005) and some forms of abiotic stress such as osmotic stress and heat stress (Jakab *et al.*, 2005). This remarkably wide range of effectiveness of BABA-induced resistance (BABA-IR) suggests that multiple resistance responses are involved. Indeed, Zimmerli *et al.* (2000) demonstrated that BABA-IR against the oomycete *H. parasitica* was still functional in *Arabidopsis* genotypes impaired in SA-dependent signalling, whereas BABA-IR against *Pst* DC3000 was blocked in these *Arabidopsis* genotypes. Hence, expression of BABA-IR involves both SA-dependent and SA-independent resistance mechanisms, and the importance of these mechanisms varies according to the nature of the challenging pathogen.

By screening previously characterised *Arabidopsis* mutants for BABA-IR, ABA was identified as an additional regulator of BABA-IR against the necrotrophic fungi *A. brassicicola* and *Plectosphaerella cucumerina*. Mutants impaired in SA, JA and ET signalling as well as camalexin production remained unaffected in BABA-IR against these fungi (Ton and Mauch-Mani, 2004). These findings suggested a novel role for ABA in the regulation of induced resistance against fungal pathogens. The role for ABA in BABA-IR was further confirmed by the identification of the mutant *ibs3* (*impaired in BABA-induced sterility 3*). This mutant is affected in the transcriptional regulation of the ABA biosynthetic gene *ABA1* and concomitantly fails to express

wild-type levels of BABA-IR against the oomycete *H. parasitica* (Ton *et al.*, 2005).

11.3 Crosstalk between defence signalling pathways

Recent genomics research has revealed that the capacity of plants to respond to the enormous diversity of attackers and beneficial organisms is highly flexible (Verhagen *et al.*, 2004; De Vos *et al.*, 2005; Sanchez *et al.*, 2005; Kempema *et al.*, 2007). The signalling networks that are activated by the plant in response to parasitic, herbivorous and beneficial organisms overlap, indicating that the regulation of the adaptive response of the plant is finely balanced between protection against aggressors and acquisition of benefits. The signalling pathways that are controlled by endogenous accumulation of defence signals such as SA, JA and ET regulate different defence responses that are effective against partially distinct classes of attackers. For many years, it was assumed that pathogens with a biotrophic lifestyle are predominantly inhibited by SA-dependent defences, whereas necrotrophic pathogens and herbivorous insects are resisted by JA/ET-dependent defences (Thomma *et al.*, 1998; Kessler and Baldwin, 2002; Glazebrook, 2005). However, over the past years it became clear that there are exceptions to this partition (Thaler *et al.*, 2004), suggesting additional layers of regulation to the plant's defensive response. In fact, plants react with a surprisingly specific response to attack by different pathogens or insects which is reflected by a highly specific amplitude and timing in the production of defence signalling compounds such as SA, JA and ET. It is thought that this so-called signal signature contributes to the specificity of the plant's primary induced defences (De Vos *et al.*, 2005). However, cross-communication between the corresponding pathways has been put forward as an additional mechanism by which plants fine-tune their defence responses (Reymond and Farmer, 1998).

11.3.1 Crosstalk between SA and JA signalling

A well-characterised example of defence-related signalling crosstalk is the interaction between the SA and JA response pathways (Rojo *et al.*, 2003; Bostock, 2005; Beckers and Spoel, 2006). Although most reports indicate a mutually antagonistic interaction between SA- and JA-dependent signalling (Bostock, 2005), synergistic interactions have been described as well (Schenk *et al.*, 2000; Van Wees *et al.*, 2000; Mur *et al.*, 2006). As a result of negative crosstalk between SA and JA, activation of the SA response should render a plant more susceptible to attackers that are resisted via JA-dependent defences and vice versa. Indeed, trade-offs between SA-dependent resistance against biotrophic pathogens and JA-dependent defence against insect herbivory have been reported (Pieterse *et al.*, 2001; Bostock, 2005). However, comparative analysis of a large number of plant–microbe and plant–insect interactions revealed

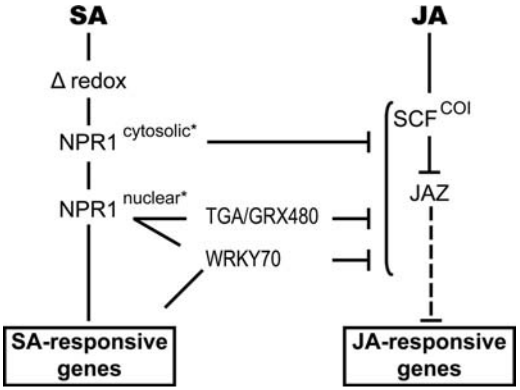


Figure 11.2 Schematic representation of molecular players in the NPR1-dependent crosstalk between salicylic acid (SA) and jasmonic acid (JA) signalling. SA-activated cytosolic NPR1 mediates downregulation of JA-responsive gene expression. The transcription factor WRKY70 and glutaredoxin GRX480 mediate suppression of JA responses in an NPR1-dependent fashion. The exact target of the JA signalling route (indicated by the extended bracket) remains to be determined. SCF^{COI}, SKP1/Cullin/F-box protein-COI1 complex; JAZ, jasmonate ZIM-domain protein. See text for details.

a more complex reality which can partially be explained by differences in experimental conditions, thereby making predictions about the outcome of such tripartite interactions difficult (Stout *et al.*, 2006).

Elucidating the molecular mechanism underlying antagonistic interactions between SA- and JA-dependent defence signalling pathways provides an excellent model to unravel the multifaceted signal interactions that shape the plant immune response. Over the past years, various regulatory components have been identified in the crosstalk between SA- and JA-dependent signalling pathways. These include proteins with stimulatory or repressive functions in either SA-dependent signalling (NPR1, WRKY70 and GRX480) or JA-dependent signalling (MPK4 and SSI2). Although different individual components have been placed in the 'crosstalk signalling network' (Fig. 11.2), their exact position and role in this network often remains to be determined. Below, the molecular players of SA/JA crosstalk identified to date are described in more detail.

11.3.1.1 NPR1

Several key regulatory proteins involved in pathway crosstalk have been identified. In *Arabidopsis*, SA-mediated suppression of JA-inducible gene expression is blocked in the *npr1* mutant, demonstrating that NPR1 plays a critical role in the crosstalk between SA and JA signalling (Spoel *et al.*, 2003). Using *npr1* plants expressing recombinant NPR1 protein with a glucocorticoid receptor hormone binding domain, Spoel *et al.* (2003) showed that nuclear localisation of NPR1 is not required for SA-mediated suppression

of the JA response. This indicates that the SA-induced suppression of the JA response is controlled by a novel cytosolic function of NPR1. Recently, a similar function of NPR1 in crosstalk was reported in rice (*Oryza sativa* L.). Overexpression of cytosolic *OsNPR1* suppressed JA-responsive transcription and enhanced the level of susceptibility to insect herbivory. Moreover, NPR1-dependent suppression of the JA response was no longer present in plants expressing OsNPR1 that was constitutively targeted to the nucleus (Yuan *et al.*, 2007).

11.3.1.2 WKRY transcription factors

WRKY transcription factors have been shown to play an important role in the regulation of SA-dependent defence responses (Maleck *et al.*, 2000; Wang *et al.*, 2006). Several WRKY transcription factors were implicated in the cross-communication between SA and JA signalling. In *Arabidopsis*, WRKY70 was identified as a node of convergence between SA and JA signalling when Li *et al.* (2004) showed that overexpression of WRKY70 caused enhanced expression of SA-inducible *PR* genes but concomitantly suppressed MeJA-induced *PDF1.2* expression. Hence, WRKY70 acts as a positive regulator of the SA response, but also as a repressor of the JA response. The exact position of WRKY70 in the crosstalk signalling network is, however, still unclear. On the one hand, WRKY70 acts as a downstream target of NPR1 (Li *et al.*, 2004; Wang *et al.*, 2006). On the other hand, transgenic plants overexpressing *WRKY70* in the *npr1-1* mutant background are impaired in *PDF1.2* repression, indicating functional NPR1 is required for WRKY70-dependent repression of this JA-responsive gene (Li *et al.*, 2006). Furthermore, recent studies with T-DNA insertion lines of *WRKY70* revealed rather diverse phenotypes, including increased levels of *PDF1.2* and *PR-1* gene expression (AbuQamar *et al.*, 2006; Li *et al.*, 2006; Ülker *et al.*, 2007), increased accumulation of SA (Wang *et al.*, 2006), enhanced resistance to *A. brassicicola* (Li *et al.*, 2006) and enhanced susceptibility to *Erysiphe cichoracearum* (Li *et al.*, 2006), *B. cinerea* (AbuQamar *et al.*, 2006) and *H. parasitica* (Knoth *et al.*, 2007). These data indicate that WRKY70 indeed affects both SA and JA signalling, but in a rather complex manner.

Besides WRKY70, WRKY11 and WRKY17 have also been implicated in crosstalk between SA and JA signalling. In *Arabidopsis*, double knockout mutants in *WRKY11* and *WRKY17* displayed enhanced levels of *PR-1* gene expression but decreased levels of JA-inducible gene expression. The expression of *WRKY70* was upregulated in this double mutant, suggesting that WRKY11 and WRKY17 function as negative regulators of WRKY70 (Journot-Catalino *et al.*, 2006). Recently, WRKY62 was added to the list of WRKY factors with a putative role in the crosstalk between SA and JA signalling. Mao *et al.* (2007) reported that the expression of *WRKY62* was synergistically induced by SA and JA in wild-type Col-0 plants but not in mutant *npr1-3*. Furthermore, transposon-tagged *wrky62* plants showed enhanced MeJA-induced transcription of *LOX2* (*LIPOXYGENASE 2*) and *VSP2*

(*VEGETATIVE STORAGE PROTEIN* 2), whereas overexpression of *WRKY62* resulted in suppression of these genes. These findings point to a repressive effect of WRKY62 on the JA response. Whether the observed WRKY62-dependent suppression of JA-inducible genes is actually activated by SA and dependent on NPR1 remains to be investigated.

11.3.1.3 Glutaredoxin GRX480

Another putative regulator in the crosstalk between SA and JA signalling is the glutaredoxin GRX480. Glutaredoxins catalyse thiol disulphide reductions and have been implicated in redox-dependent regulation of protein activities (Lemaire, 2004). Recently, Ndamukong *et al.* (2007) identified this glutaredoxin in a two-hybrid screen for interactors with TGA transcription factors. Expression of *GRX480* was found to be inducible by SA and dependent on NPR1. Interestingly, overexpression of *GRX480* completely abolished MeJA-induced *PDF1.2* expression but hardly affected the induction of JA-responsive LOX2 and VSP. This suggests that GRX480 regulates only a part of SA-induced suppression of the JA response. The suppressive effect of GRX480 on *PDF1.2* induction was abolished in the *tga2tga5tga6* triple mutant, indicating that the interaction between GRX480 and TGA transcription factors is essential for the GRX480-dependent crosstalk (Ndamukong *et al.*, 2007). These results suggest a model in which NPR1 induces GRX480, which in turn interacts with TGA transcription factors to suppress JA-responsive gene induction.

11.3.1.4 MPK4

Apart from NPR1-dependent crosstalk between SA and JA signalling pathways, several NPR1-independent routes have also been described. Petersen *et al.* (2000) identified MAP kinase 4 (MPK4) as a negative regulator of SA signalling and positive regulator of JA signalling in *Arabidopsis*. Inactivation of MPK4 resulted in elevated SA levels and constitutive *PR* gene expression. MPK4 was shown to function upstream of SA accumulation, but independently of NPR1 (Petersen *et al.*, 2000). Inactivation of MPK4 in mutant *mpk4* resulted in enhanced susceptibility to *A. brassicicola*, which is sensitive to JA-dependent defences. Moreover, *mpk4* blocked JA-responsive gene expression independently of SA accumulation, as *mpk4*/NahG transgenics still exhibited increased susceptibility to *A. brassicicola* and suppression of MeJA-induced *PDF1.2* expression (Petersen *et al.*, 2000; Brodersen *et al.*, 2006). Thus, MPK4 is required for JA-responsive gene expression. EDS1 and PAD4 were identified as downstream effectors of MPK4 function, having the opposite effect of MPK4 by behaving as activators of SA signalling and repressors of JA signalling (Brodersen *et al.*, 2006). Another target of MPK4 is its substrate MKS1 (MAP kinase 4 substrate 1). Phosphorylation of MKS1 is thought to repress SA signalling, since *MKS1*-RNAi could partially rescue the *PR-1*-overexpressing phenotype of *mpk4*. However, over- or underexpression of MKS1 did not affect *PDF1.2* gene expression, indicating that other downstream targets of MPK4 are involved in JA signalling. MKS1 interacted with two WRKY

transcription factors, WRKY25 and WRKY33, that could both be phosphorylated by MPK4 (Andreasson *et al.*, 2005). These WRKYs might be downstream targets of MPK4 that contribute to the repression of SA responses, as overexpression of both WRKY25 and WRKY33 resulted in decreased pathogen-induced *PR-1* expression and enhanced susceptibility to *P. syringae* (Zheng *et al.*, 2006, 2007).

11.3.1.5 SSI2

Mutant *ssi2* (*suppressor of SA insensitivity 2*) is defective in stearoyl ACP desaturase, resulting in an altered fatty acid content. Also, this mutant shows *NPR1*-independent constitutive *PR-1* expression and enhanced resistance to *H. parasitica* but is impaired in *PDF1.2* transcription and resistance to *B. cinerea*. Inhibition of *PDF1.2* is not dependent on elevated SA levels, since *ssi2*/NahG plants were still unable to express JA-induced *PDF1.2* (Kachroo *et al.*, 2001). Mutations that restored the lowered 18:1 fatty acid levels rescued the *ssi2* mutant phenotype, suggesting a role for fatty acid signalling in SA/JA crosstalk (Kachroo *et al.*, 2003, 2004).

11.3.1.6 Targets of the SA/JA antagonism

By analogy to the inhibitory effect of aspirin (acetyl salicylic acid) on the production of the octadecanoid prostaglandin in mammalian systems, evidence suggests that the antagonistic effect of SA on the JA response in plants is targeted at the level of octadecanoid biosynthesis (Pan *et al.*, 1998). Several reports have described suppression of JA biosynthesis genes by SA or its acetylated form, suggesting that SA targets the octadecanoid biosynthesis pathway to suppress downstream JA signalling (Peña-Cortés *et al.*, 1993; Doares *et al.*, 1995; Spoel *et al.*, 2003). However, it is not known whether suppression of the JA biosynthesis pathway is essential in transducing the SA antagonism. Recent evidence shows that *Arabidopsis* mutants that are blocked in JA biosynthesis have normal levels of SA-mediated suppression of MeJA-induced *PDF1.2* expression, suggesting that downstream components in the JA signalling pathway are targeted by SA (H.A. Léon Reyes and C.M.J. Pieterse, unpublished results). In view of its importance in the JA signalling pathway, it is tempting to speculate that the SCF^{COI1} complex is the target of SA in SA/JA crosstalk. Alternatively, downstream components of COI1 might be involved in the SA/JA antagonism. The recently identified JAZ repressor proteins can also be an attractive target for the SA-mediated suppression of JA signalling. SA-mediated stabilisation of these repressors would inhibit JA signalling and a broad-spectrum effect on JA target genes would be achieved.

11.3.2 Crosstalk between other defence signals

Besides crosstalk between SA and JA signalling, other phytohormone pathways have been shown to interact and affect each other's downstream

defence responses. In *Arabidopsis*, for example, JA-inducible defence mechanisms against pathogens and insects are antagonistically co-regulated by ET and ABA via MYC2 and ERF1 (ETHYLENE RESPONSE FACTOR 1), respectively (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004). These transcription factors regulate divergent branches of the JA signalling route that are involved in the response to wounding and pathogen attack, respectively. MYC2-dependent gene induction is synergistically induced by JA and ABA, while ERF1-dependent gene induction is controlled by the combined action of JA and ET. These findings show that the antagonistic crosstalk between ABA and ET signalling not only influences developmental process (Pierik *et al.*, 2006) but also contributes to the fine-tuning of defence against pathogens and insects (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004).

11.3.3 Decoys of plant defences

Crosstalk between defence pathways is thought to provide the plant with a powerful regulatory potential that helps it to prioritise and 'decide' which defensive strategy to follow, depending on the type of attacker. Yet, it seems that attackers and beneficial organisms have also evolved to manipulate plants for their own benefit by shutting down induced defences or modulating the signalling network (Pieterse and Dicke, 2007). Some microbial pathogens have acquired the ability to manipulate the plant's signalling infrastructure by producing phytohormones or their functional mimics to 'trick' the plant into activating inappropriate defences (Robert-Seilaniantz *et al.*, 2007). For instance, coronatine is a phytotoxin produced by virulent *P. syringae* strains (Nomura *et al.*, 2005) that functions as an extremely potent mimic of JA-Ile. It is assumed that the coronatine from the pathogen triggers a hypersensitive induction of JA-Ile responses, causing suppression of SA-dependent defences through crosstalk (Brooks *et al.*, 2005; Cui *et al.*, 2005). Recently, coronatine was also demonstrated to prevent PAMP-induced stomatal closure, thereby facilitating bacterial entry into the leaf (Melotto *et al.*, 2006).

Insects can also induce ineffective plant signalling cascades as a decoy mechanism. For instance, *Bemisia tabaci* (silverleaf whitefly) nymphs trigger SA-responsive gene expression and, as a consequence, suppress the induction of effectual JA- and ET-dependent genes (Zarate *et al.*, 2007). Further analysis of mutant and transgenic *Arabidopsis* lines revealed that JA-regulated rather than SA-dependent defences contributed to basal resistance against silverleaf whitefly nymphs. Hence, the nymphs are capable of exploiting negative crosstalk between SA and JA to make plant tissue more accessible for infestation (Zarate *et al.*, 2007). Recently, egg-derived elicitors from *Pieris brassicae* and *P. rapae* have been suggested to suppress JA-dependent responses through mechanisms of SA-induced crosstalk as an insect strategy to benefit hatching larvae (Little *et al.*, 2007).

11.4 Priming for enhanced defence

Different signal signatures, pathway crosstalk and attacker-mediated suppression of host defence signalling are major molecular mechanisms by which the defence response of the plant is shaped. Priming for enhanced defence adds another layer of complexity to the way plants can adapt to their biotic environment. The primed state can be induced biologically by beneficial rhizobacteria, mycorrhizal fungi, pathogens or insect herbivores, but also chemically, for example, by exogenous application of low doses of SA, JA or BABA (Conrath *et al.*, 2002, 2006). In primed plants, defence responses are not activated directly by the priming agent but are accelerated following perception of biotic or abiotic stress signals, resulting in an enhanced level of resistance (Conrath *et al.*, 2002, 2006). By studying the costs and benefits of priming in *Arabidopsis*, it was shown that the fitness costs of priming are lower than those of constitutively activated defences such as expressed in the constitutive SAR-expressing mutant *cpr1* (*constitutive expresser of PR genes*; Van Hulten *et al.*, 2006). The fitness benefits of priming outweighed its costs under pathogen pressure, suggesting that priming functions as an ecological adaptation of the plant to respond faster to a hostile environment.

11.4.1 Priming during SAR

Remarkably, first implications for the involvement of priming in SAR arose from studies using elicitors of chemical-induced resistance. Low amounts of BTH and SA did not directly activate defence responses but rather accelerated the expression of *PAL* and *PR* genes (Mur *et al.*, 1996; Kohler *et al.*, 2002). Mutant analyses demonstrated a role for NPR1 in priming of SA-mediated defences. Besides being blocked in direct activation of defence genes, *npr1* plants were not able to prime the expression of *PAL* for a faster response to *Pst* DC3000 when pretreated with BTH or an avirulent strain of *Pst* DC3000 (Kohler *et al.*, 2002). Hence, NPR1 is involved in priming for enhanced expression of SA-mediated defences. Also, BTH-primed deposition of callose-containing papillae at *H. parasitica* penetration sites was disrupted in *npr1* (Fig. 11.3a). Together with antimicrobial components residing in the callose matrix, these papillae may form a physical and/or chemical barrier to certain pathogens, preventing them from invading the plant tissue.

Besides *npr1*, *edr1* (*enhanced disease resistance 1*) that is mutated in an *MAPKKK* (Frye *et al.*, 2001) has an altered priming phenotype. The enhanced protective level against various pathogens of *edr1* is not based on constitutive activation of defence responses (Frye and Innes, 1998; Van Hulten *et al.*, 2006). Rather, *edr1* plants are constitutively primed for augmented expression of diverse defences such as *PR-1* expression, callose deposition and HR (Frye and Innes, 1998; Van Hulten *et al.*, 2006), suggesting that the EDR1 protein is a repressor of priming.

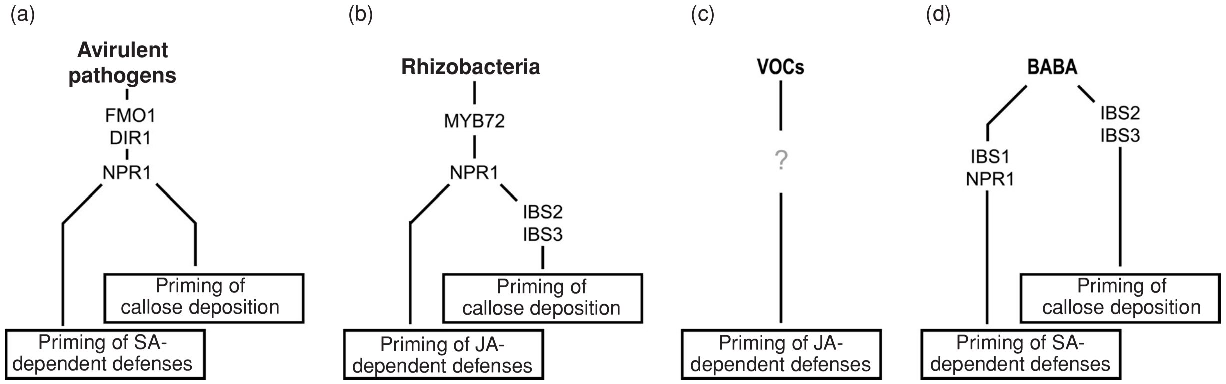


Figure 11.3 Priming pathways for enhanced defence in *Arabidopsis*. (a) Priming during systemic acquired resistance (SAR). The SAR response requires FMO1 and DIR1 for propagation of the systemic signal and requires intact NPR1 for both priming of enhanced SA-dependent defence gene expression and priming of increased callose deposition. (b) Priming during induced systemic resistance (ISR). Rhizobacteria-mediated ISR requires MYB72 and NPR1 and leads to priming of enhanced JA-dependent defence gene expression and priming of accelerated callose deposition via the IBS2 and IBS3 proteins. (c) Priming by VOCs. Volatiles released upon insect herbivory prime plants for both direct and indirect JA-dependent defence responses. (d) Priming by β-aminobutyric acid (BABA). BABA can prime for both enhanced SA-dependent defence gene expression and increased callose deposition upon pathogen attack. NPR1 and IBS1 are required for priming of SA-dependent responses, but not for priming of callose deposition. Rather, IBS2 and IBS3 are needed for priming of increased formation of callose-rich papillae. SA, salicylic acid; JA, jasmonic acid; VOCs, volatile organic compounds.

11.4.2 Priming during ISR

In contrast to SAR, ISR is not associated with direct induction or priming of *PR* gene expression (Van Wees *et al.*, 1999). To detect ISR-induced genes in systemic tissue of *Arabidopsis*, Verhagen *et al.* (2004) analysed the transcriptome of leaves upon colonisation of the roots by ISR-inducing WCS417r rhizobacteria. Despite elevated levels of resistance, no differences in gene expression could be observed between the distal parts of ISR- and control-treated plants prior to pathogen infection. However, a similar analysis of induced systemic tissue after pathogen infection led to the identification of a set of genes that responded faster and stronger to pathogen attack (Verhagen *et al.*, 2004), which was in line with earlier observations of selected defence-related marker genes (Van Wees *et al.*, 1999). In particular, genes regulated by JA or ET exhibited a primed response upon pathogen attack, corresponding with the requirement of intact JA and ET signal transduction pathways for the expression of WCS417r-mediated ISR. Similarly, ISR-inducing *P. putida* LSW17S was demonstrated to prime JA- and ET-dependent defence responses of *Arabidopsis* (Ahn *et al.*, 2007). In many other interactions between plants and plant growth-promoting rhizobacteria, increased resistance arises from a potentiated expression of defence-related genes (Benhamou *et al.*, 1996; De Meyer *et al.*, 1999; Ahn *et al.*, 2002; Kim *et al.*, 2004; Tjamos *et al.*, 2005). Recent findings suggest that WCS417r-mediated ISR in *Arabidopsis* against the oomycete pathogen *H. parasitica* is based on an augmented deposition of callose-rich papillae. This priming response was dependent on NPR1 as well as the IBS2 and IBS3 proteins that play a role in BABA-IR against fungi and oomycetes (S. van der Ent and J. Ton, unpublished results) (Fig. 11.3b).

11.4.3 Priming by airborne signals

Priming by airborne signals such as VOCs produced following insect herbivory is a major topic in molecular ecological research on plant–herbivore and plant–plant interactions (Baldwin *et al.*, 2006; Turlings and Ton, 2006). Analogous to chemicals such as INA and BTH, VOCs can either directly activate defence responses of recipient plants or prime them to respond faster and stronger to stress exposure (Baldwin *et al.*, 2006; Turlings and Ton, 2006). Engelberth and co-workers (2004) demonstrated that green leafy volatiles produced by corn plants after insect feeding prime neighbouring plants for enhanced JA-dependent defence against herbivory, rather than directly activating it (Fig. 11.3c). In a laboratory study with maize, VOCs were similarly demonstrated to prime neighbouring plants for enhanced direct and indirect defence, resulting in reduced performance of caterpillars of the Egyptian cotton leafworm *S. littoralis* (direct defence) and improved attractiveness to parasitoid *Cotesia marginiventris* wasps that feed on the insect herbivores

(indirect defence) (Ton *et al.*, 2007). Also in the field, herbivory-induced VOCs have been demonstrated to prime nearby plants for enhanced direct and indirect defence responses (Kessler *et al.*, 2006), indicating that priming has a role in plant defence under ecological conditions. Another demonstration of VOC-induced priming in nature was provided by Heil and Silva Bueno (2007). They showed that VOCs released by beetle-infested 'emitter' leaves of lima bean plants growing in their natural habitat primed nearby 'receiver' leaves for enhanced secretion of extrafloral nectar, resulting in prolonged visitation by predatory arthropods. Although the active players in VOC-mediated priming differ among plant species, it seems to be a common defence strategy in plants (Baldwin *et al.*, 2006).

11.4.4 Priming during BABA-IR

Application of high concentrations of BABA directly activates defence responses that are regulated either by SA or by ABA (Van Hulten *et al.*, 2006; J. Ton and M. Van Hulten, unpublished results). However, lower amounts of BABA prime for enhanced induction of NPR1-dependent *PR* gene expression as well as NPR1-independent deposition of callose-containing papillae at entry sites of the pathogen (Fig. 11.3d) (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). Augmented callose deposition is also involved in and even essential for BABA-IR against the fungal pathogens *A. brassicicola* and *P. cucumerina* (Ton and Mauch-Mani, 2004).

Screening for mutants that are impaired in BABA-induced sterility (*ibs*) resulted in the identification of three genes (*IBS1*, *AtSAC1b/IBS2* and *ABA1/IBS3*) with a regulatory role in BABA signalling (Ton *et al.*, 2005). While BABA-IR is also impaired in *ibs1*, *ibs2* and *ibs3*, the three mutants show normal levels of basal resistance. IBS1 is involved in the SA-dependent component of BABA-IR, while AtSAC1b/IBS2 and ABA1/IBS3 are required for ABA-regulated callose deposition and subsequent BABA-IR (Fig. 11.3d).

11.4.5 Molecular mechanisms of priming

Priming is associated with different types of induced resistance (Conrath *et al.*, 2002, 2006), and it provides the plant with an enhanced capacity for rapid and effective activation of cellular defence responses once a pathogen contacts. Such a defence system could explain the broad-spectrum action that is often associated with induced resistance. The molecular mechanisms underlying priming are still poorly understood. Hypothetically, the primed state could be based on the accumulation or post-translational modification of one or more signalling proteins that, after being expressed or modified, still remain inactive. Upon subsequent perception of a stress, a second signalling event could 'hyperactivate' the signalling protein, triggering a potentiated signal

transduction. Another hypothesis suggests that priming enables accelerated defence gene expression by inducing the accumulation of essential transcription factors. After stress recognition, signal transduction in primed cells then could directly induce an appropriate set of defence genes, thereby avoiding a preliminary step of transcription factor expression.

11.5 Concluding remarks

Plant diseases and pests are responsible for large crop losses in agriculture. Conventional crop protection is based on resistance breeding and application of chemical agents. Classic resistance breeding depends on the availability of *R* genes that often have limited durability. The use of chemical agents and their persistence in soil are potentially harmful to the environment, especially when chemicals are applied repeatedly in large amounts such as in the control of soilborne fungal pathogens. Moreover, these disease control strategies are directed against a single or a small group of plant pathogens. Induced disease resistance might be an attractive alternative form of plant protection, as it is based on the activation of extant resistance mechanisms in the plant and is effective against a broad spectrum of plant pathogens (Kuc, 1982; Van Loon *et al.*, 1998).

Knowledge of defence signalling pathways has been proven to be instrumental in the development of new strategies for broad-spectrum disease resistance. Examples are genetic engineering of the SAR pathway and the development of defence signal-mimicking chemicals such as BTH. However, crosstalk between SA- and JA-dependent defence pathways may be a burden when enhanced pathogen resistance is associated with reduced resistance against insects.

Priming fits well in the ecological context of induced resistance. While constitutive activation of inducible defences involves major costs that affect plant growth and reproduction (Heil, 2002), priming for enhanced defence is associated with significant fitness benefits when disease occurs (Van Hulten *et al.*, 2006). Consequently, plants in the primed state are effectively protected against stress without major trade-off effects on commercially and ecologically important traits, such as growth and seed set. Thus, from an economic perspective, priming could be the plant's solution to the trade-off dilemma between disease protection and the cost of defence activation.

Future research on the molecular mechanisms of induced resistance, crosstalk between plant defence pathways and priming for enhanced defence will provide more insight into how plants are able to integrate signals into appropriate defences cost-effectively. Ultimately, this will not only provide a deeper understanding of how plants cope with different enemies, but also be instrumental in developing strategies for biologically based, environmentally friendly and durable crop protection.

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